



# The Role of Non-Classical Regulatory T Cells in HIV-1 Infection

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## **The Role of Non-classical Regulatory T Cells in HIV-1 Infection**

### **Abstract**

Regulatory T cells represent a specialized subpopulation of T lymphocytes that may modulate spontaneous HIV-1 disease progression by suppressing immune activation or inhibiting antiviral T cell immune responses. While effects of classical CD25<sup>hi</sup>FoxP3<sup>+</sup>CD4<sup>+</sup> regulatory T cells during HIV-1 infection have been analyzed in a series of recent investigations, very little is known about the role of non-classical regulatory T cells that do not express intracellular FoxP3. Here I evaluated two groups of non-classical Treg cells. One is phenotypically identified by the surface expression of HLA-G, an HLA class Ib molecule. The other Treg cell population is characterized by the surface expression of latency-associated peptide (LAP), a membrane-bound form of TGF- $\beta$ . Both HLA-G and LAP-expressing T cells are present in small proportions in peripheral blood of healthy individuals.

I performed a systematic study on the phenotypic and functional profile of HLA-G- and LAP- expressing regulatory T (Treg) cells in patients with different stages of HIV-1 infection. I found that HLA-G-expressing Treg cells were highly susceptible to HIV-1 infection, and were significantly reduced in individuals with progressive HIV-1 disease courses. Moreover, the proportion of HLA-G<sup>+</sup> CD4 and CD8 T cells was positively correlated with CD4 T cell count and inversely correlated with markers of HIV-1 associated immune activation. Mechanistically, this correlation corresponded to a substantially increased ability of HLA-G<sup>+</sup> Treg cells to inhibit bystander immune

activation, while only minimally affecting functional properties of HIV-1-specific T cells. In contrast, no significant change in LAP<sup>+</sup> Treg cell frequencies was found in progressive HIV-1 infection, and these frequencies were not correlated with immune activation. This observation was consistent with functional analysis, which indicated that LAP<sup>+</sup> Treg cells did not suppress bystander activation. These investigations indicate an important role of HLA-G<sup>+</sup> Treg cells for balancing bystander immune activation and anti-viral immune activity in HIV-1 infection, and suggest that the loss of these cells during advanced HIV-1 infection may contribute to immune dysregulation and HIV-1 disease progression. In the meantime, LAP<sup>+</sup> Treg cells do not appear to play an important role in determining HIV-1 disease outcome.

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## Attributions

This work has been accepted as Chun Li, Ilona Toth, Julian Schulze zur Wiesch, Florencia Pereyra, Jennifer Rychert, Eric S. Rosenberg, Jan van Lunzen, Mathias Lichterfeld, Xu G. Yu. HLA-G<sup>+</sup> regulatory T cells reduce bystander immune activation in HIV-1 infection. *PLOS Pathogens*, in press.

I performed the experiments presented in this dissertation, with the exceptions listed below.

Lymph node staining was performed by Ilona Toth, Julian Schulze zur Wiesch and Jan van Lunzen.

## Abbreviations

AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
CTL	cytotoxic T lymphocyte
EC	elite controller
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
LILRA	leukocyte immunoglobulin-like receptor, subfamily A
LILRB	leukocyte immunoglobulin-like receptor, subfamily B
LNMC	lymph node mononuclear cell
LTNP	long-term nonprogressor
LTR	long terminal repeat
mDC	myeloid dendritic cells
MHC	major histocompatibility complex
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
prog	progressor
RT	reverse transcriptase
SIV	simian immunodeficiency virus
VC	viremic controller

# **Chapter 1**

## **Introduction**

## **HIV-1/AIDS global epidemic**

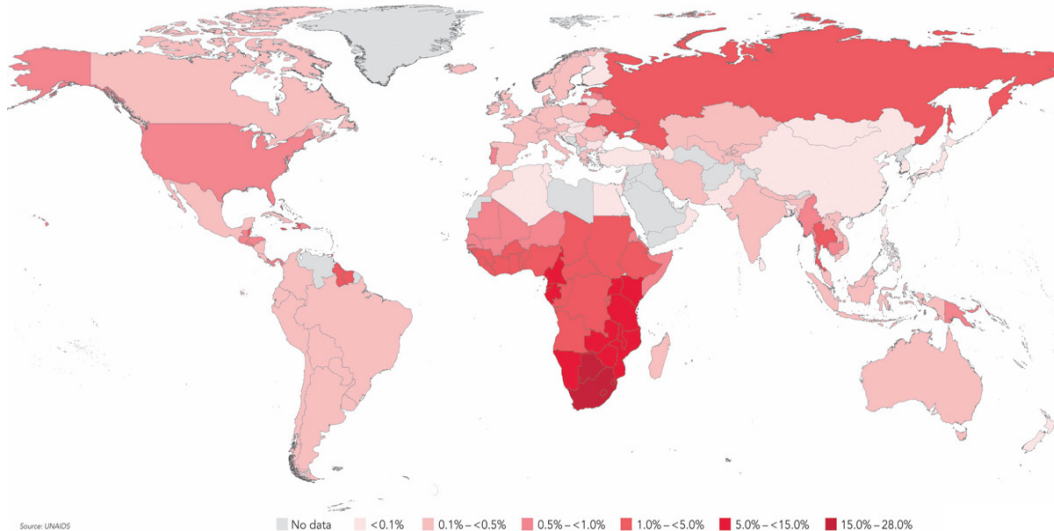
The first case of HIV/AIDS infection was reported in 1981, when four homosexual men who presented with pneumocystis carinii pneumonia and mucosal candidiasis were found to have severely reduced helper T cells (1). Two years later, the “human T-cell leukemia virus” was isolated in AIDS patients (2, 3), and subsequently named human immunodeficiency virus (HIV) (4).

Since then, the rapid pandemic spread of HIV/AIDS has made it one of the most pressing threats to global public health. It has been estimated that, in 2010, 34 million people were living with HIV (5), 10% of whom were children under 15 years of age. Despite extensive efforts in prevention and treatment, 2.7 million people acquired the disease in 2010 and 1.8 million people were killed. HIV/AIDS has its strongest impact in sub-Saharan Africa, where 25.8 million people are HIV positive, and new infections occurs at a high rate of 1.7 million per year (6). In the US, 1.2 million individuals are living with the disease, and incidence rate is approximately 50,000 per year (Figure 1-1) (7). In addition, the virus is spreading rapidly in Latin America, eastern Europe, central, south and south-east Asia, with an infected population of more than 1 million in each region (Figure 1-1) (7). Furthermore, because HIV/AIDS disproportionately affects young and mid-aged individuals, AIDS-related deaths lead to significant loss of human capital, and seriously compromise economic development.



## 2010: A global view of HIV infection

33.3 million people [31.4–35.3 million] living with HIV, 2009



**Figure 1-1.** Global distribution of HIV-1-infected populations. The HIV pandemic has a wide impact all over the world, with its most significant influence in Sub-Saharan Africa. *Source: UNAIDS 2010.*

Two subtypes of HIV are currently identified – HIV subtype 1 (HIV-1) and HIV subtype 2 (HIV-2). Both viruses were transferred to humans from non-human primates in west-central Africa: HIV-1 originated in chimpanzees, and HIV-2 originated from sooty mangabeys (8-10). While HIV-1 serves as the primary cause of AIDS and has a worldwide presence, HIV-2 is less transmittable and pathogenic (9, 11), and its endemic is limited to West Africa and Asia.

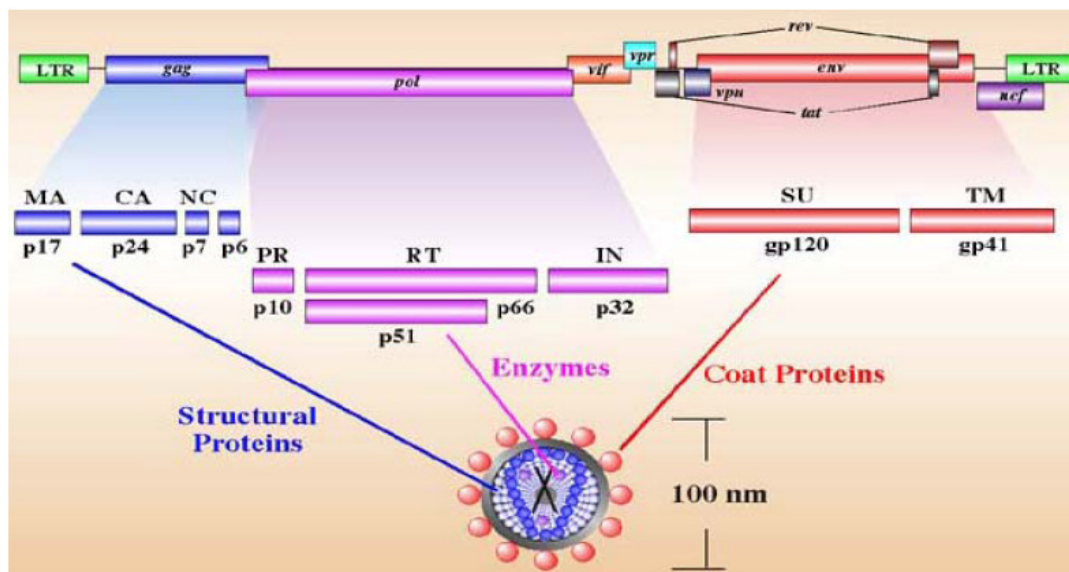
### Molecular biology of HIV-1

HIV-1 belongs to the Lentivirus genus of the *Retroviridae* family. A mature HIV-1 virion contains two copies of 9.7kb, positive-sense, single-stranded RNA genome. The genome is packed within an inner conical-shaped core, which is further

surrounded by an outer envelope derived from the host cell plasma phospholipid bilayer. The HIV-1 genome encodes a long terminal repeat (LTR) and nine viral genes: three structural genes - *gag*, *pol*, and *env*, and six accessory genes - *tat*, *rev*, *vif*, *nef*, *vpr*, *vpu* (Figure 1-2) (12). The three structural genes each encode a large poly-protein that goes through post-translational cleavage to yield smaller functional subunits. Briefly, the Gag polyprotein comprises four proteins that build the physical structure of the virus-capsid protein p24, matrix protein p17, and nucleocapsid proteins p6 and p7 (13). These subunits are involved in viral packaging and uncoating processes (13). The Pol precursor yields the catalytic proteins protease, reverse transcriptase, and integrase (14); and the Env product is comprised of a gp160 protein, which is further cleaved into a gp41 transmembrane subunit and a surface gp120 subunit. The six accessory genes each produce one protein required for optimizing viral replication and pathogenicity (15, 16).

The HIV-1 replication cycle starts when a virion attaches to a host cell through interactions between the viral envelope gp120 and the CD4 receptor on the host cells and subsequently, to a chemokine coreceptor (typically CCR5 or CXCR4). Coreceptor binding in turn induces structural changes in gp41, which extends its N-terminal fusion peptide into the cell membrane and triggers fusion of the viral and cellular membranes. Membrane fusion allows the release of the viral core into the cytoplasm, which subsequently disassociates to expose the viral nucleoprotein complex. The HIV RNA genome is then reverse transcribed into a double-stranded DNA through a series of RNA-dependent or DNA-dependent DNA synthesis processes. This double-stranded DNA forms a pre-integration complex, which is transported to the cell nucleus by the viral protein Integrase (Int) and integrates into the host genome. The integrated provirus serves as template for the transcription of viral RNAs, catalyzed partially by

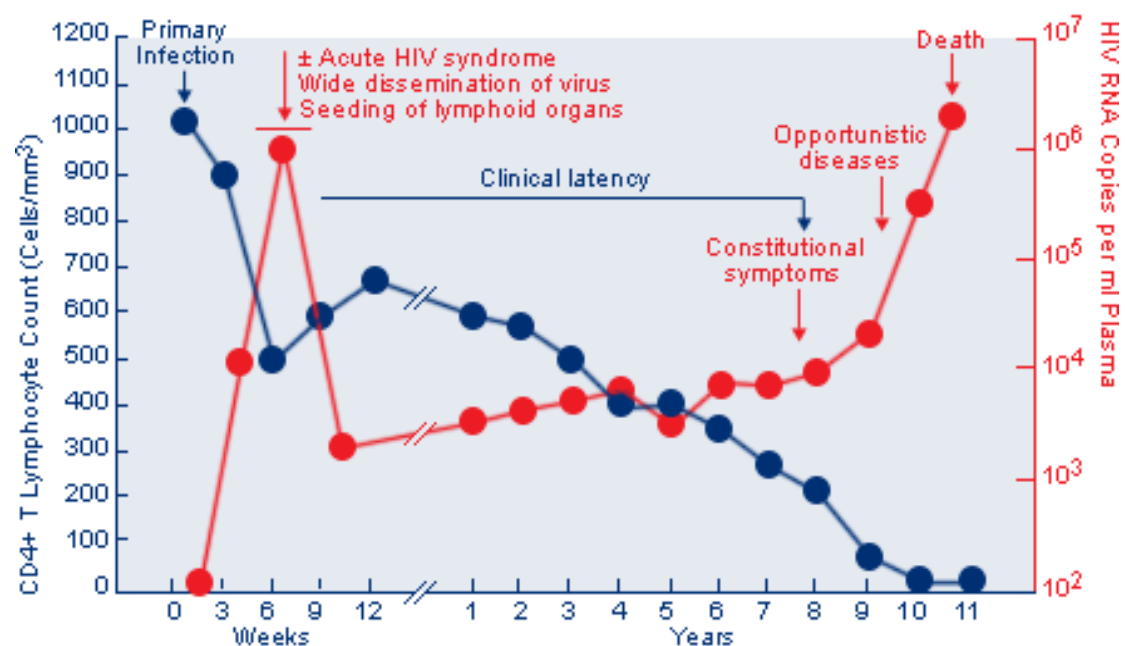
Tat. There are two categories of HIV RNA transcripts – the unspliced RNA is designated for packaging into new virions, and spliced mRNA is used for production of viral proteins by the host translational machinery. As the new virions are assembled and bud from the cell membrane, the Gag and Pol polyproteins are processed to their functional subunits, a process called “maturation”.



**Figure 1-2.** Genomic organization of HIV-1. Adapted from Stewart SA, et al: *Human Immunodeficiency Virus Type 1 Vpr Induces Apoptosis through Caspase Activation. J Virol*, Vol. 74, No. 7: 3105-3111 (2000).

### HIV-1 natural history

On average it takes about 8 to 10 years upon HIV-1 infection for clinical manifestation of AIDS to develop. Based on the viral replication and host immunity status, the overall process can be divided into 3 stages: acute infection, clinical latency and systemic immune deficiency (Figure 1-3).



**Figure 1-3.** Natural history of HIV-1 infection. The whole infection course can be divided into primary infection, clinical latency and advanced disease according to viral replication status and host CD4 T lymphocyte counts. *Adapted from Pantaleo G, et al: The immunopathogenesis of human immunodeficiency virus infection. N Engl J Med (1993).*

### Acute infection

The first stage, termed acute or primary infection, spans from the infection point to the point where anti-HIV antibody can be detected. During this stage, the virus enters the body through blood or mucosal linings, is engulfed by the local immune cells and transferred into regional lymph nodes, where a high concentration of CD4 T cells reside. HIV rapidly replicates in the lymph nodes, and gets disseminated into the circulation, resulting in a burst of viral load. Peak viremia is usually associated with clinical symptoms of seroconversion, and over a few weeks declines to a “set point”, which is maintained throughout the clinical latency period.

The onset of clinical symptoms generally occurs within 2 to 6 weeks post virus-exposure. These are nonspecific and temporary symptoms, such as fever (most

common), pharyngitis, skin rash, nausea and dry cough (17). For this reason, often times people may not seek immediate medical attention till they progress to more advanced stages.

### ***Clinical latency***

The second stage of HIV infection has been termed the clinical latency, during which patients typically do not display symptoms due to their infection. Despite the decline in viral load to a set point, new virions are continually produced at  $10^9$  -  $10^{10}$  per day. These virions actively infect CD4 T cells, causing a rapid turnover and eventually depletion of this subset in the blood, gastrointestinal tract, and lymph node (18). Once CD4 count falls below the clinical critical point of 200 cells/ $\mu$ l, the risk of opportunistic infections increases dramatically, and a diagnosis of AIDS is made.

### ***Advanced disease***

Before the age of highly active antiretroviral therapy (HAART), patients in the advanced-disease stage were likely to develop AIDS-related illnesses and die within two years of the AIDS diagnosis (17). When CD4 counts fall below 200 cells/ $\mu$ l, patients are susceptible to classic AIDS-related events such as *Pneumocystis jirovecii* pneumonia and Kaposi's sarcoma; in addition, diseases such as cytomegalovirus retinitis, CNS non-Hodgkin's lymphoma are much more likely to develop once CD4 counts fall below 50 cells/ $\mu$ l (19). Furthermore, these diseases often co-exist in a host, making diagnosis and treatment even more challenging (17).

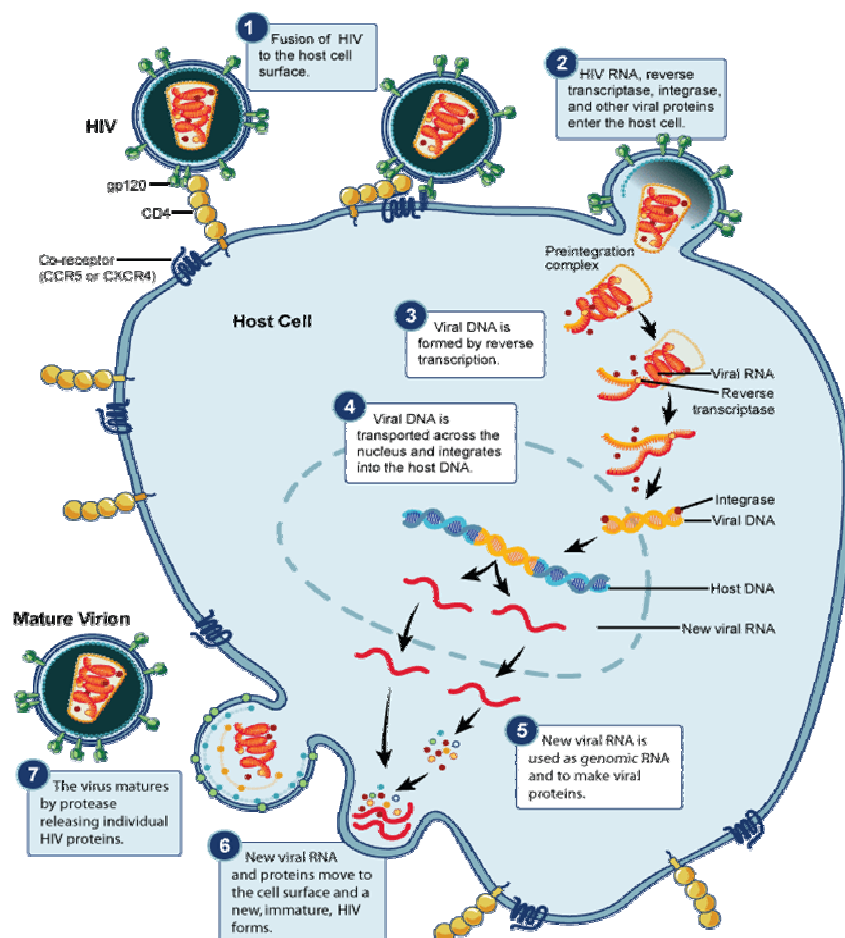
### **HIV-1/AIDS treatment (HAART)**

The first antiretroviral drug, Azido-thymidine (AZT, also known as ZDV), was introduced more than two decades ago. Since then a variety of antiretroviral agents have been developed and are widely used. Owing to the unique potency and side-effect profiles of each drug, as well as the rapid emergence of viral drug resistance, the most effective strategy consists of a combination of drugs to maximize the suppressive effects by simultaneously targeting multiple steps in the viral life cycle. Such combination therapy has been termed HAART (highly active antiretroviral therapy).

Antiretroviral drugs are typically categorized by the viral life-cycle step they inhibit (Figure 1-4). Although a detailed description of the various antiretroviral drugs is beyond the scope of this thesis, selected aspects of some of the most widely used drugs will be briefly discussed.

The first step in the HIV life cycle is the attachment and fusion of the virus with host cell membrane. Fusion inhibitors are designed either as cellular receptor/co-receptor antagonists that prevent the attachment of viral envelope protein gp120, or as peptides that inhibit post-fusion conformational changes of gp41 (17). By blocking this early step, these drugs effectively abrogate viral infection.

Reverse transcription is a central step in viral replication cycle. Reverse transcriptase inhibitors comprise nucleoside/nucleotide analogue reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI). The chemical structures of NRTI drugs often closely resemble natural nucleosides except that they lack a 3'-hydroxyl group on the deoxyribose moiety, which is essential for the reverse transcriptional elongation. NNRTIs, on the other hand, are chemicals with longer half-lives than the NRTIs and bind directly to the reverse transcriptase to interfere with its catalytic activities (17).



**Figure 1-4.** HIV life cycle. HAART regimens target a variety of viral replication steps, including fusion with the cell membrane (step 1), reverse transcription (step 2), integration (step 4), and maturation (step 7). *Source: National Institute of Allergy and Infectious Diseases (NIAID). <http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/hivreplicationcycle.aspx>.*

Integration is a pivotal step in the HIV-1 life cycle, where the virus inserts the reverse transcribed genome into the host chromatin. Inhibiting viral replication by blocking the viral integrase that catalyzes this step is an attractive therapeutic strategy. High throughput screening has identified the 4-aryl-2,4-diketobutanoic acid inhibitors containing a distinct diketo acid moiety (DKA) to be an attractive approach (20). These

DKA compounds bind to conserved motif of integrase and inhibit its enzymatic activities (21).

HIV-1 protease is responsible for the posttranslational processing of the viral Gag and Pol polyproteins to yield their functional subunits (22). Failure to process the polyprotein results in immature and non-infectious viral particles (23-25). Based on the structure of protease, researchers design inhibitors by substituting the scissile amid bond in the original sequence with hydroxyethylene dipeptide isosteres; the later represents a transition-state analogue that binds to HIV-1 protease with a higher affinity than its natural substrate (26-28). Other small-molecule protease inhibitors have been identified based on computer-assisted search of crystallographic databases for structures that would dock into the active site of the enzyme (26).

### **Important prognostic markers**

It is critical for both prognostication and therapeutic intervention to predict the disease progression and outcome for HIV infection, since it features a prolonged period of clinical latency. Up until now, the two prognostic factors that have yielded the most accurate predictions of disease progression are the CD4 lymphocyte count and the plasma viral load after seroconversion (29-32). In addition, quantitation of immune activation levels has recently emerged as a strong predictor of the disease outcome (33, 34).

Immune activation reflects the “fighting” mode of the immune system and is crucial for pathogen clearance. That being said, over-activation of the immune system often results in increased susceptibility and faster turnover rate of CD4 T cells (35), as well as enhanced apoptosis of other players in both innate and adaptive immune responses (36-38). Furthermore, abnormal high immune activation leads to severe



disruption of the vascular endothelium and other tissues, by driving high-level, disorganized secretion of pro-inflammatory cytokines and chemokines (39, 40). In a chronic inflammatory disease such as HIV-1-infection, even in the setting of HAART, the damage of persistent immune activation often outweighs its benefit, and high activation levels (as measured by the surface expression of CD38 and/or HLA-DR) are consistently observed to be associated with faster disease progression (33, 34, 41, 42).

A similar association between chronic immune activation and disease progression is also found in primates infected with AIDS viruses. Simian immunodeficiency virus (SIV) is the primate HIV-1 counterpart. It infects rhesus macaques and results in HIV-like syndromes; untreated animals eventually progress to AIDS. However, when SIV infects its natural host, the sooty mangabey, the host does not develop any SIV-related syndromes despite high level viremia. Interestingly, unlike rhesus macaques, which display massive chronic activation and enhanced activation-induced apoptosis, sooty mangabeys exhibit minimal immune activation (43). This significant divergence in immune activation status is believed to contribute to the different disease outcomes between the two species (43).

To better understand immune activation in HIV-1 infection, we need to know what is causing it. At first glance, it may appear to be the direct result of viral infection. However, the fact that the majority of dying CD4 cells are not productively infected suggests that this is not the primary reason (38, 44). A few mechanisms have been proposed. Firstly, HIV gene products can induce activation of lymphocytes and macrophages, which in turn secrete pro-inflammatory cytokines (45-47). Secondly, HIV-1-related co-infections such as CMV and EBV can contribute to the activation of the immune system (48-50). Thirdly, HIV-1 causes disruption of the intestinal mucosal barrier, which in turn leads to translocation of gut microbial products into the circulation

(51). Further, a recent study discovered a large number of previously undescribed enteric viruses in the gastrointestinal tract of SIV-infected rhesus monkeys (52). The activities of these viruses can contribute to intestinal leakage and systemic immune activation (52). In any of the situations mentioned above, cells are not productively activated through specific interactions between HIV antigens and the cellular T cell receptors (TCR). Instead, they are stimulated either by non-HIV antigens, or by antigen-independent mechanisms, such as surface receptor ligation, or soluble factors secretion. The later non-TCR-mediated, non-antigen-specific activation is termed bystander activation (38, 53). Indeed, the majority of dying CD4 T cells in HIV-1 infection appear to be the uninfected “bystander” cells (38, 44). The bystander killing involves actions of both viral gene products such as Tat, Vpr, and Nef (54, 55), and also host-derived factors, including tumor necrosis factor- $\alpha$ , Fas ligand and TRAIL (56, 57). These mechanisms are useful to explain the death of the cells that are not even entered by the virus. Recently, Doitsh and colleagues discovered an interesting mechanism for the post-viral-entry bystander cell death (58). Briefly, reverse transcription is initiated but failed to reach completion in these non-productively-infected cells (59). The long reverse transcript intermediates accumulate stably in the cell (60), and are able to induce coordinated pro-apoptotic and pro-inflammatory responses (58).

### **HIV-1 elite control**

HIV elite controllers (ECs), or elite suppressors, originally refer to HIV-infected individuals that maintain viral load under the detection limit for a prolonged period of time in the absence of HAART (61-63). With the advances in RNA detection technologies, researchers are now able to detect a single copy of HIV RNA per milliliter

of blood; the cut-off for elite controllers remains defined as viremia below 50 RNA copies/ml (64). Based on this definition, the EC subgroup consists only about 0.3% of the general HIV-infected population (64).

Clinical investigations have indicated that disease progression and for HIV transmission is significantly delayed for patients who maintain viral load below 2,000 copies/ml (65), and the subset of patients with viral load between 50 copies/ml and 2,000 copies/ml is defined as “viremic controllers” (64). This viremia level serves as a predictor of disease outcome, and also as a goal for disease treatment or vaccine design.

HIV controllers (especially elite controllers) are significantly different from untreated chronic progressors in a number of aspects, including but not limited to the facts that they have CD4 T cells with reduced susceptibility to HIV-1 (66), fully functional CD8 T cells targeting to conservative viral epitopes (67, 68), and potent antigen presenting dendritic cells (69). This indicates the complex and heterogeneous nature of elite controllers, and suggests that mechanisms for HIV control are multifactorial (70).

As a closely related concept, persons categorized as long-term non-progressors (LTNPs) are often studied. Unlike HIV controllers, LTNP is defined solely based on the clinical outcome: it refers to patients who do not show clinical evidence of immune deficiency in the absence of antiretroviral therapy for more than 10 years (71). As this definition does not take into consideration of viremia, one of the most fundamental driving forces of disease progression, it is sometimes less accurate in predicting disease outcome (72). As a matter of fact, LTNPs tend to vary widely in viral loads, and as high as 90% of them eventually lose their maintenance of stable CD4 cell counts and progress (72).

## **Mechanisms for HIV-1 control**

Representing the “functional cure” of HIV-1 infection, elite controller studies have been moving into the center of the HIV field. Mechanisms for the elite control phenotype have been extensively investigated around the areas of virus dynamics, host genetics, and host immune responses (62, 71).

### ***Virus dynamics***

HIV can rapidly escape from detection by the immune system, particularly from cytotoxic T cells (CTLs) and/or natural killer cells (NKs). However these mutations are often generated at the cost of reducing viral fitness, defined by how well a virus can replicate and survive in a particular host environment (73). It is therefore possible that elite controllers are capable of eliciting strong HIV-specific immune responses that render the virus less fit. Indeed, we discovered that recombinant viruses derived from EC *pol* sequence (encodes reverse transcriptase and integrase) featured a significantly reduced replication capacity than that derived from chronic progressors (74), suggesting an involvement of attenuated virus in the elite control phenotype. Another possibility along the same line is that the transmitted founder viral strain in EC is already weakened (75, 76).

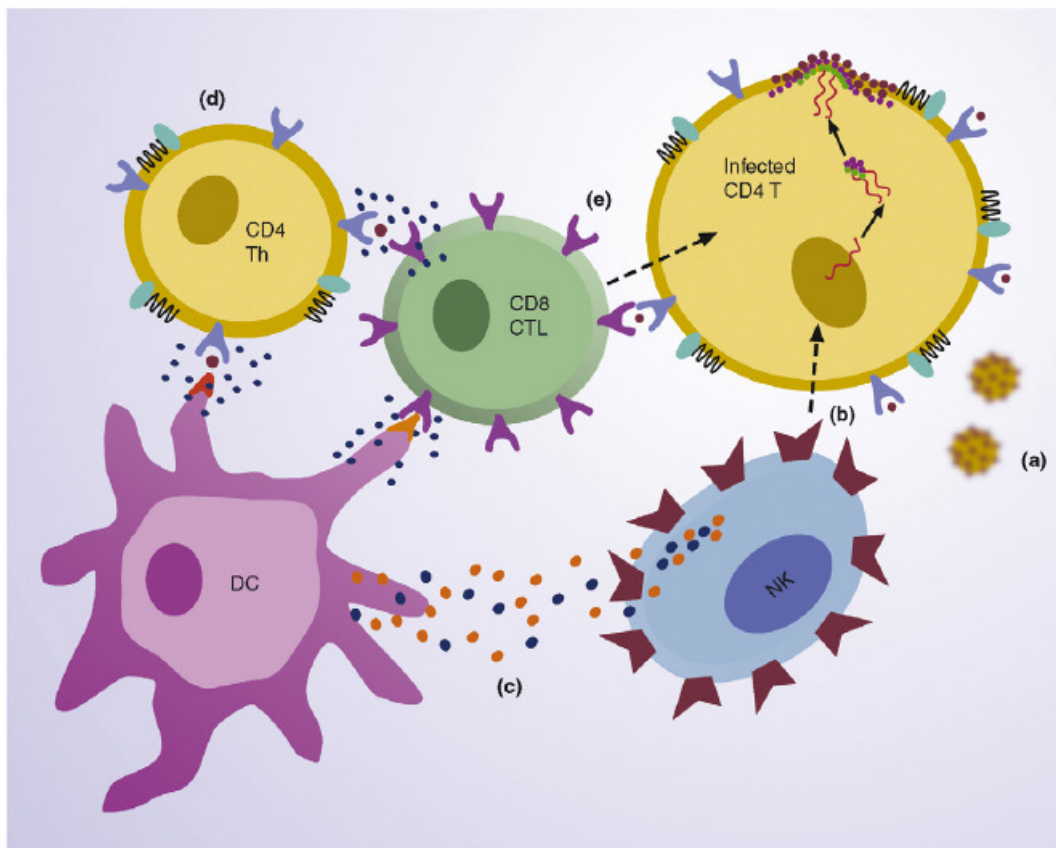
### ***Host factors***

Because HIV-1 relies heavily on the host cellular machinery for its survival, host molecules involved in the viral life cycle could affect or even abrogate viral replication. An example involves a small host protein, p21, which was previously known to play an important role as a tumor-suppressor factor (77). We discovered that CD4 T cells from ECs were more resistant to HIV-1 infection than that of HIV-1 seronegative individuals

or progressors (66). Moreover, this resistance was strongly associated with an elevation in p21 expression (66). Further mechanistic studies demonstrate that p21 was able to interact with and block a cycline-dependent kinase (CDK), which was crucial for HIV transcriptional elongation (66).

### ***Host immune response***

While HIV immune control has been primarily attributed to adaptive immunity, innate immunity also makes significant contribution in determining disease outcomes. Their individual roles are summarized in Figure 1-5. For the focus of this thesis, we will primarily discuss the roles of T cells in regulating the disease progression.



**Figure 1-5.** Cross talks between innate immunity and adaptive immunity during HIV-1 infection.

**Figure 1-5 (continued).** (a) HIV-1 infecting CD4 T cells (b) NK cell directly killing infected cells (c) dendritic cells secreting cytokine to boost an effective immune response (d) CD4 T cells helping to maintain HIV-1-specific CD8 T cell responses. (e) HIV-specific CTL effectively killing infected CD4 T cells. *Source: Saez-Cirion, et al: HIV controllers: how do they tame the virus? Trends Immunol. 28(12): p. 532-40 (2007).*

### **CD8 cytotoxic T lymphocytes (CTLs)**

A number of *in vivo* and *ex vivo* experiments have established the central role of CD8 T cells in controlling HIV-1 infection (78). It was shown that transfer of CTLs provided full protection against HIV-1 challenge in mice with severe combined immune deficiency (SCID) (79). In rhesus macaques, transient CD8 T cell depletion resulted in a 100-10,000-fold increase in viral burden, and return of CD8 T cells reduced high viremia (80). In humans, favorable disease outcomes are consistently correlated with strong CD8 anti-viral activities (81, 82), including high functional avidity (83, 84), strong proliferative capacity (81), polyfunctionality (85, 86), strong capacity to produce cytotoxic proteins (81, 87, 88), preferential targeting of specific viral proteins (89, 90), and production of IL-21 (91). Phenotypically, HIV-1-specific CTLs from EC are generally more differentiated (71), less activated (49, 92) and less exhausted (93) than those from progressors.

The anti-viral activities of CD8 T cells can be broadly classified into noncytotoxic effects (94-96), and HLA-class I- restricted cytotoxic T cell responses (82, 85, 97, 98). The non-cytotoxic effects were first shown by Walker et al, who demonstrated that CD8 cells from HIV controllers were able to suppress HIV replication up to >90% in *ex-vivo* infected CD4 T cells, via secretion of soluble factors (99). In addition, CD8 T cells from EC were shown to directly kill infected cells without exogenous stimulation (82).

Consistent with a dominant effect of HIV-1-specific CD8 T cell responses on HIV-1 immune control, HLA class I polymorphisms critically affect HIV disease

outcome (100, 101). Two large-scale studies have consistently shown a strong association between homozygosity at HLA-A/HLA-B loci and rapid disease progression (102, 103). A possible explanation for this finding is that heterozygotes have the ability to present a broader range of viral peptides, so that the time for the virus to develop an escape mutation is significantly longer than that for homozygotes (100). The other main mechanism by which HLA I may influence HIV disease outcome is through the expression of specific polymorphic alleles which can either grant the host “protection” against disease progression, or render the host more susceptible to progression. While HLA-B\*57, and B\*58 alleles are well-recognized protective HLA alleles, HLA-B\*35-Px, B\*801 and B\*702 are often associated with faster disease progression. Interestingly, despite being one of the strongest indicators of disease outcome, the expression of a protective allele alone does not predict immune protection. We have consistently observed that certain proportions of HIV controllers with one or more protective alleles ultimately become progressors in our Boston HIV cohorts (66, 74). Many theories have been developed to explain the mechanism of protective alleles. For example, the protection associated with HLA-B\*57 expression has been attributed to the improved capability of CD8 T cells to target a diverse pool of HIV-1 peptides (104, 105), to their improved ability to effectively inhibit virus’ escape by cross-recognizing dominant Gag variants (106), and to their suppression of virus production from super-infected CD4 T cells (98).

### **CD4 helper cells**

In contrast to CD8 T cells, functional properties of CD4 T cells during HIV-1 infection are more complicated and less well defined. On the one hand, CD4 T cells can critically contribute to optimized innate and adaptive immune responses directed

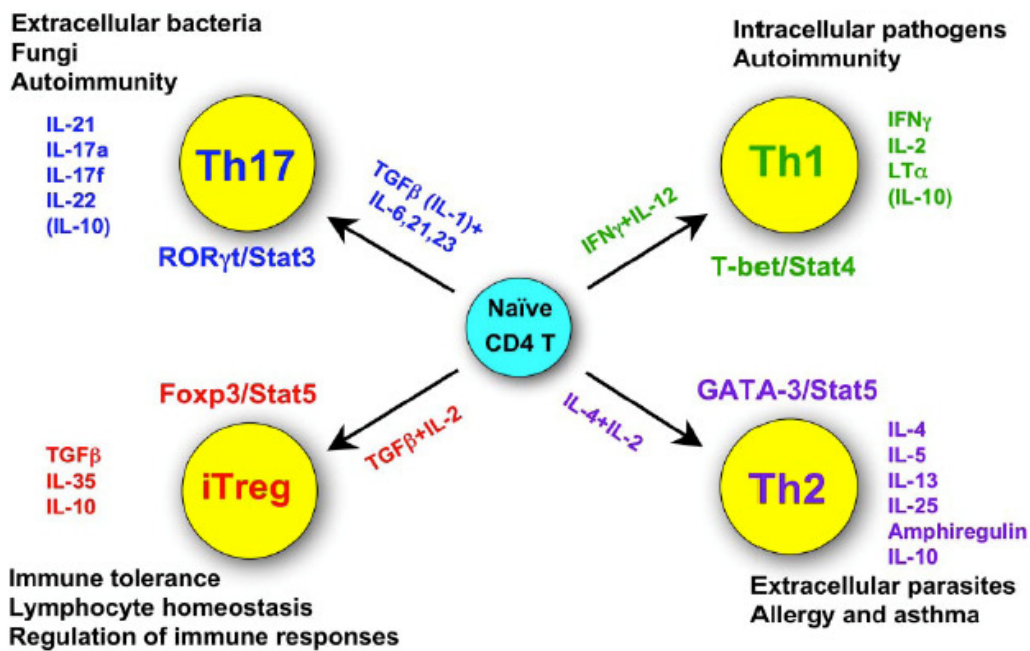
against HIV-1; on the other hand, activated CD4 T cells are preferentially infected by the virus and serve as the “fuel to burn” (107). Unambiguous definition of CD4 T cells’ net effect in HIV-1 infection is vital for understanding the pathogenesis of the disease, and for designing cell-based HIV/AIDS vaccines.

During the acute phase of HIV-1 infection, strong CD4 T cell expansion predicts a low viremia set point and favorable disease outcomes (108), whereas failure to do so is an important indicator for HIV/AIDS progression (107).

During the chronic latency stage, delayed disease progression has been correlated with enhanced proliferative capacity (90, 109), strong cytokine secretion (89) (109-112), strong cytotoxic effect (108), greater IL-21 production (113, 114), higher avidity and TCR binding affinity (115), and lower expression of immune inhibitory molecules of HIV-1-specific CD4 T cells (116). These functional capacities enable CD4 T cells to directly contribute to viral inhibition, and/or help maintain effective and long-lasting CD8 T cell and B cell responses (117, 118). Indeed, *ex vivo* human studies have shown that the depletion of HIV-1-specific CD4 T cells led to loss of the proliferative CD8 lymphocytes, which can be restored by adding back autologous CD4 cells (119). Furthermore, preliminary data from the recent RV144 Thailand vaccine trial indicated an positive association between protection from the infection and HIV-1 specific CD4 T cells (120). Similar correlations between SIV-specific CD4 T cells and disease outcomes have been observed in primate studies (121). Taken together, these results suggest that eliciting effective HIV-1-specific CD4 T cell responses protect the host from HIV-1 disease progression. However, the fact that CD4 T cells are the primary target cells for HIV-1, and therefore support viral replication and contribute to viral persistence (107), must be taken into consideration for the future design of cell-mediated immune therapies.



Besides regulating the adaptive immunity, CD4 T cells also actively modulate the innate immune responses, mainly through secretion of cytokines. Indeed, CD4 T cells are further categorized into subsets based on their cytokine secretion profile and their implication in the immune response against different pathogens (Figure 1-6): Th1 cells mediate pro-inflammatory responses and are characterized by predominant production of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ); Th2 cells are associated with anti-inflammatory activity, and mainly secrete interleukin-4 (IL-4), IL-5 and IL-13; Th17 cells produce high levels of IL-17 and stimulate inflammatory responses in organs and tissues infected with parasites; regulatory T (Treg) cells are specialized in suppressing immune responses.



**Figure 1-6.** CD4 T helper cell categories based on associated transcriptional factors, products and functions. *Source: Zhu, J. and Paul, W. E., CD4 T cells: fates, functions, and faults. Blood, 112(5): p. 1557-69 (2008).*

### Regulatory T (Treg) cells

The recognition of pathogens by immune cells triggers a number of subsequent events including activation, expansion, differentiation, migration, and mediation of effector immune responses. The overall process is positively regulated, aiming to eliminate the pathogen as fast as possible. However, excessive immune activation causes serious damage to the cells and tissues, and is deleterious to the host. Therefore mechanisms have evolved to stop the inflammation after successful removal of the pathogen. Regulatory T cells play this important role of regulating immune responses.

Regulatory T cells are a subset of CD3 T cells that are capable of suppressing potentially deleterious activities of conventional T cells (122). They are essential components of the immune system to maintain peripheral tolerance. As a result, significant reduction of Treg cell number or impaired functionality is frequently associated with autoimmune diseases or inflammatory disorders, such as type 1 diabetes, asthma and inflammatory bowel disease (123-127). Although Treg cells are important for preventing excessive inflammatory reactions, they can also block beneficial responses by making the immune system insensitive to certain pathogens (128, 129), or limiting anti-tumor activities (130).

### Treg cell markers

Currently most Treg cell studies have been focused on the classical CD25<sup>hi</sup>Foxp3<sup>+</sup>CD127<sup>lo</sup>CD4<sup>+</sup> Treg population. These Treg cells were first found in mice by their constitutive expression of CD25 and low expression level of CD127 (131-133). A few years later, a similar population of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells was isolated from peripheral blood, thymus, lymph nodes and cord blood in humans (134-138). Follow up studies found that the differentiation and function of these Treg cells was

tightly controlled by the expression of a single transcriptional factor, FoxP3 (Forkhead box P3) (139). The lack of FoxP3 expression was correlated with severe autoimmune disease in mice and in humans (140, 141); whereas ectopic expression of FoxP3 confers strong suppressor function to naïve CD25<sup>hi</sup>CD4 T cells (142, 143).

Despite being the most important regulator, FoxP3's expression is not exclusive to Treg cells (144). In addition, more markers have been identified to be co-expressed by CD25<sup>hi</sup>FoxP3<sup>+</sup>CD127<sup>lo</sup>CD4<sup>+</sup> T cells, including but not limited to CTLA-4 (145), CD62L (146), CD103 (147) and GARP (148, 149). However, none of these markers are necessary or sufficient to define a homogenous regulatory T cell population (150-154).

Most recently, novel populations of T cells with immunosuppressive properties have been identified. Unlike the classical ones, these non-classical Treg cells do not express or depend on FoxP3 to achieve suppression, but rather use other cell-associated or soluble molecules to exert immunoregulation (153, 154). For this thesis, we focused on the roles of two non-classical Treg cells - HLA-G- and LAP expressing T cells – in HIV-1 infection.

HLA-G is a non-classical MHC class 1b molecule, that can exist both in membrane-bound and soluble forms (155). It regulates both innate and adaptive immune responses by interacting with immune inhibitory receptors. Through these interactions, HLA-G can reduce the cytotoxicity of natural killer cells and CD8 T cells, in addition to suppress CD4 T cell alloproliferation and dendritic cell maturation (156-159). HLA-G's expression is largely limited to certain tissues and organs such as placental trophoblastic tissues, thymus, cornea, nail matrix and pancreas under physiological conditions (160). However, ectopic expression of HLA-G is frequently associated with organ transplantation, autoimmune diseases, viral infections and cancer (161, 162).

Within these pathological contexts, HLA-G can either play a positive role by allowing better acceptance of organ graft or limiting autoimmune responses, or play a negative role by permitting malignant or virus-infected cells to escape from immunosurveillance. HLA-G's functional properties in HIV-1 infection are not clear.

TGF- $\beta$  critically contributes to the differentiation, maintenance and function of natural Treg cells (163-166). Latency-associated peptide, or LAP, is a propeptide that covalently binds to TGF- $\beta$  and keeps it in a latent status. LAP-expressing CD4 T cells are shown to be hypoproliferative, and suppressive of poly-clonal T cell proliferation in a Foxp3-independent manner (153).

#### Mechanisms for suppression

The general mechanisms employed by the CD25<sup>hi</sup>FoxP3<sup>+</sup>CD127<sup>lo</sup>CD4<sup>+</sup> Treg cells include secretion of inhibitory cytokines, cytolysis, metabolic disruption, and modulation of dendritic-cell maturation and function (167).

Inhibitory cytokines, such as interleukin-10 (IL-10) and TGF- $\beta$ , have been consistently observed to be associated with suppressive effects of classical Treg cells. IL-10, produced primarily by monocytes but tightly regulated by Treg cells, suppresses the antigen presentation and phagocytosis potency in monocytes/macrophages (168), as well as the Th17-cell mediated inflammation (169). In mice, a deletion in the IL-10 gene resulted in over exuberant immune responses to the malaria parasite *Plasmodium chabaudi* (170). Moreover, these mice became susceptible to inflammatory bowel disease (170). In human allergy and asthma models, it was also suggested that IL-10 was indispensable for Treg-cell mediated disease control (171, 172). TGF- $\beta$ 's role, on the other hand, is slightly more controversial. Although cell-

culture studies indicated that TGF- $\beta$  was not necessary for Treg cells' suppressive activities using a neutralizing antibody (173, 174), more evidence supported a critical role of the surface TGF- $\beta$  in maintaining immunoregulatory properties by Treg cells, both *in vitro* and *in vivo*. These studies indicated that blocking TGF- $\beta$  signal led to complete abrogation of cell-contact mediated suppression (175, 176). Moreover, TGF- $\beta$  was shown to convert naïve Foxp3<sup>-</sup> CD4 T cells to CD25<sup>+</sup> CD4 regulatory T cells by inducing expression of FoxP3 (177, 178). In addition to IL-10 and TGF- $\beta$ , IL-35 (a member of IL-12 heterodimeric cytokine family) has been recently described to be preferentially expressed by Treg cells, and serves to maximize their suppressive capacity (179). Abrogation of IL-35 severely reduced the regulatory activities of Treg cells, and ectopic expression conferred suppressive ability to naïve T cells (179).

Besides secreting inhibitory cytokines, Treg cells are also able to kill target cells, a function that was previously thought to be limited to CTLs and NKs. Moreover, Treg cells express high levels of surface CD25, and are thought to cause deprivation-induced apoptosis of targeting cells by consuming local IL-2 (180-182). Besides directly affecting T cells, Treg cells can also down-regulate DC function, which in turn influences effector T cells (183-185).

### Treg cells and HIV-1 infection

Treg cells serve as a “double-edge-sword” in regulating HIV-1 disease progression. On the one hand, they may limit the beneficial immune responses (186, 187); and on the other hand, they could suppress harmful, sometimes detrimental immune activation (188, 189). In this thesis, we focused on the dynamics of Treg frequencies during natural disease courses. Up to now, most reports support an

enhanced frequency of the classical CD25<sup>+</sup>FoxP3<sup>+</sup>CD4<sup>+</sup> Treg cells in the peripheral blood during HIV infection (150, 190-192), though it is still controversial (187, 193). Despite different opinions on the change in relative proportions of Treg cells, it is generally agreed that the absolute Treg cell counts are reduced in chronic progressors (150, 187, 188, 192, 193). Collectively, these results suggest that HIV affects Treg cells to a lesser extent than conventional T cells, and that Treg cell preservation might be associated with a better disease outcome. Possible explanations for the relative preservation of Treg cells include better survival, increased proliferation, and tissue redistribution of this subset, as well as increased peripheral conversion from conventional T cells (194).

Furthermore, we analyzed the functional impact of non-classical Treg cells on HIV-1 disease outcomes. For this purpose, we compared the immune activation level in an *in vitro* system in the presence and absence of Treg cells. We also evaluated changes, upon adding Treg cells, in several important functional aspects that are directly associated with HIV-1 disease outcomes. Two such factors are antigen-specific proliferative capacity and effector cytokine secretion (195-197). As early as in 1997, Rosenberg and colleagues demonstrated a direct correlation between HIV-1-specific CD4 T cell responses (both proliferation and Interferon- $\gamma$  production) and control of virus replication and delayed disease progression (109). This association has been confirmed by several studies involving larger patient cohorts (90). CD8 proliferative capacity is also correlated with disease outcome: maintenance of HIV-1-specific proliferation was shown to be inversely associated with viremia in a large cohort study (198), and strong baseline HIV-1-specific CD8 proliferation was prospectively associated with a slow disease progression (197). These beneficial antigen-specific T cell proliferative responses may be influenced by Treg cells (187). In addition, Treg

cells may also affect production of important anti-viral cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-2 (IL-2) (186).

### **Current investigations**

The classical Treg cells decline at a slower rate than conventional CD4 T cells during progressive HIV-1 infection, and this suggests that these cells may play an important role in the immune pathogenesis of HIV-1 infection. Indeed, functional data from previous studies have demonstrated that classical Treg cells can potentially suppress HIV-1-specific T cell responses (186, 187), and in this way may contribute to the failure of achieving T cell mediated immune control of HIV-1 replication. However, classical Treg cells may also have beneficial effects on HIV-1 disease progression by reducing the deleterious consequences of HIV-1 associated immune activation (188, 189). Recently, HLA-G- and LAP-expressing Treg cells have been identified that differ from classical Tregs by the lack of intracellular FoxP3 expression. However the numeric distribution and functional properties of either Treg population during HIV-1 infection is not known.

In the present study, we systematically analyzed the expression and function of HLA-G- and LAP-expressing Treg cells in patients with different stages of HIV-1 disease infection. Our results indicate a profound reduction of HLA-G<sup>+</sup> CD4 Treg cells in individuals with progressive HIV-1 infection that may stem from a higher susceptibility of these cells to HIV-1 infection, and functionally contribute to HIV-1-associated immune over-activation.

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## **Chapter 2**

# **Comprehensive characterization of HLA-G- expressing regulatory T cells in HIV-1 infection**

## Introduction

Several Treg populations identified recently differ from classical CD25<sup>hi</sup>CD127<sup>lo</sup>FoxP3<sup>+</sup> Treg cells by the lack of intracellular expression of FoxP3. One group of such non-classical Treg cells is defined by the surface expression of human leukocyte antigen (HLA-G) (1). HLA-G represents a non-classical major histocompatibility complex (MHC)-1b. It differs from the classical MHCs in the following aspects:

**1) Low polymorphisms.** While classical HLA-ABCs have hundreds of isoforms, HLA-G's primary transcript yields only seven protein isoforms, including both membrane-bound (HLA-G1, G2, G3, G4) and soluble proteins (HLA-G5, G6, G7). Among these, HLA-G1 and HLA-G5 are the dominant isoforms in expression and function (2), and are the focus of most HLA-G studies as well as this thesis.

**2) Limited expression profile.** While classical HLAs are expressed by cells and tissues throughout the body, HLA-G expression is largely restricted to placental trophoblastic tissues in healthy individuals (3). However under pathological conditions, such as autoimmune diseases, cancer and transplantation, enhanced or ectopic expression of HLA-G can be detected on T cells, monocytes and dendritic cells (1).

**3) Distinctive functionality.** Instead of promoting antigen presentation and T cell priming which is the primary function of classical HLAs, HLA-G is more involved in an immunoregulatory role (1). HLA-G<sup>+</sup> Treg cells can suppress T cell activation in a contact independent manner, and their regulatory effects are reversible following neutralization with HLA-G blocking antibodies (4).

HLA-G interacts primarily with immune inhibitory receptors, such as leukocyte immunoglobulin-like receptor subfamily (LILRB1, LILRB2), and killer cell

immunoglobulin-like receptors (KIR2DL4) (2). LILRB1 has a wide presence in all myeloid and lymphoid cells (5), while LILRB2 is restricted to myeloid lineage (6). KIR2DL4 is mainly found in CD56<sup>hi</sup> NK cells and some T cell lines (7, 8).

Previous reports suggested that the proportion of HLA-G expressing lymphocytes was increased during HIV-1 infection (9), however, such investigations were conducted in unselected populations of HIV-1 positive persons, and did not address the functional role of HLA-G positive T cells during different stages of HIV-1 disease progression.

In the present study, we have systematically analyzed the expression and function of HLA-G-expressing Treg cells in patients with different stages of HIV-1 infection. Our results indicate a profound reduction of HLA-G<sup>+</sup> Treg cells in individuals with progressive HIV-1 disease that may stem from a higher susceptibility of these cells to HIV-1 infection, and functionally contribute to HIV-1-associated immune overactivation.

## **Materials and Methods**

### *Study participants*

HIV-1-infected patients and seronegative individuals were recruited according to protocols approved by the Institutional Review Board of the Massachusetts General Hospital in Boston. Samples of mononuclear cells extracted from lymph nodes and peripheral blood were obtained from HIV-1 infected patients recruited at the University of Hamburg (Germany) according to a protocol approved by the local Ethics Committee.

We have included in the study 5 patient groups with various status of HIV infection: 22 patients with primary infection and seroconversion within 3 months prior to recruitment (acutely infected patients), 28 treatment-naïve HIV-1 infected individuals with chronic progressive infection (progressors), 26 HIV-1 infected persons successfully treated with highly active antiretroviral therapy (HAART-treated), 24 patients with spontaneous control of HIV-1 replication (controllers), and 26 non-HIV-infected healthy individuals (HIV negative).

Among acutely-infected patients, the viral load ranges from 36,600 to 2,790,000 copies/ml, with a median of 99,900 copies/ml; CD4 T cell count varies from 265 to 1047/ $\mu$ l with a median of 475/ $\mu$ l. For chronic progressors, the viremia varies from 20,187 to 685,000 copies/ml, with a median of 48,215 copies/ml; CD4 T cell count varies from 204 to 652/ $\mu$ l, with a median of 396/ $\mu$ l. As for HAART-treated patients, all of them have viremia <50 copies/ml; CD4 T cell count ranges from 242 to 1493/ $\mu$ l with a median of 402/ $\mu$ l. For controllers, the viral load <1,000 copies/ml, and CD4 T cell count varies from 347 to 1879/ $\mu$ l, with a median of 924/ $\mu$ l.

### *Immunophenotypic analysis*

Peripheral blood mononuclear cell (PBMC) were isolated from whole blood using Ficoll density centrifugation. Lymph node mononuclear cell (LNMC) were extracted from freshly-excised lymph node samples according to routine procedures (10). LNMC or PBMC were stained with LIVE/DEAD cell viability dye (Invitrogen) and monoclonal antibodies directed against CD4, CD25, CD127, CD45RA, CCR7 (BD Biosciences), CD57 and PD-1 (Biolegend), CD8 (Invitrogen), HLA-G (clone MEM-G/9, Abcam), and when indicated, LILRB1 (clone HP-F1, ebioscience). After incubation for 20 minutes at room temperature, cells were fixed with PBS containing 0.5% fetal calf serum and 1% formaldehyde. Antigen-specific staining was done by pre-incubating HIV-, CMV-, EBV-, or influenza-specific class I tetramer or pentamer with PBMCs on ice for 30min, washing away unbounded tetramer, followed by a regular surface staining.

For FoxP3 intracellular staining, anti-human FoxP3 Alexa Fluor 647 (ebioscience) was used with foxp3 staining buffer set (ebioscience) per manufacturer's instruction. Briefly,  $1 \times 10^6$  PBMCs were stained by a panel of surface markers including anti-CD3 (PE-Cy7), anti-CD4 (Alexa-Fluor 700), anti-CD8 (Pacific Blue), anti-CD25 (PE-Cy5), anti-HLA-G (PE) and cell viability dye. After 20 min incubation at room temperature, cells were washed by PBS then by staining buffer, and incubated in fixation/permeabilization buffer at room temperature for 30 min. After being fully permeabilized, cells were washed once by staining buffer and twice by permeabilization buffer, and stained with Foxp3-Alexa Fluor 647 for 30 min at room temperature. They were then washed twice with permeabilization buffer and resuspended in staining buffer for flow cytometry analysis. Cells were acquired on LSR Fortessa™ Cell Analyzer (BD Biosciences) using DiVA software. Data were analyzed using FlowJo software (Tree Star).

### *Western blots*

CD3 T cells were negatively isolated from HIV seronegative donors. To isolate HLA-G<sup>+</sup> cells, samples were incubated with PE anti-HLA-G antibody (clone MEM-G/9) for 30 min on ice, followed by immunomagnetic depletion of HLA-G using anti-PE magnetic beads according to the manufacturer's protocol (Miltenyi). Cells bound to the column and in the flow through were collected as HLA-G<sup>+</sup> and HLA-G<sup>-</sup> portion, respectively. Equal amounts of HLA-G<sup>+</sup> and HLA-G<sup>-</sup> cells were cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM Hepes, 10% heat-inactivated FCS and 50U/ml IL-2, for a period of 4 days. Supernatant and cell lysate were collected.

Equal amounts of supernatant or cell lysate were subjected to SDS-PAGE (Life Technologies), electroblotted, incubated with HLA-G antibodies (clone 4H84, Abcam) in 5%-milk PBS, followed by visualization with horseradish peroxidase (HRP)-labeled secondary antibodies and enhanced chemiluminescence (ECL) detection reactions (Amersham Biosciences) according to standard protocols.

### *Cell isolation and sorting*

CD4 or CD8 T cells were isolated using a negative cell purification kit (Stemcell), according to the manufacturer's instructions. Cell purity was > 95%. Subsequently, fluorescence activated cell sorting was used to divide bulk CD4 T cells into three subgroups: HLA-G<sup>-</sup> CD25<sup>hi</sup> CD4 T cells, HLA-G<sup>+</sup> CD25<sup>-</sup> CD4 T cells, and HLA-G<sup>-</sup> CD25<sup>-</sup> CD4 T cells. For this purpose, CD4 cells were labeled with respective monoclonal antibodies and viability dye, washed, and sorted on a FACSAria instrument (BD Biosciences) at 70 pounds per square inch. For isolation



of CD8 Treg subsets, purified bulk CD8 T cells were sorted into three T cell subsets: HLA-G<sup>+</sup> CD25<sup>-</sup> CD8 T cells, CD25<sup>hi</sup> CD28<sup>-</sup> CD8 T cells and a control cell population of HLA-G<sup>-</sup> CD25<sup>-</sup> CD8 T cells, using similar sorting conditions.

#### *Proliferation Assay*

Isolated PBMCs from HIV-1 infected individuals were stained with 0.25 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) and mixed with sorted autologous Treg populations or control T cells without regulatory activity at ratios of 4:1. Afterwards, cells were stimulated with a pool of overlapping peptides spanning the clade B consensus sequences of HIV-1 gag (2µg/ml per peptide), a pool of overlapping peptides spanning the entire sequence of human CMV pp65 (2µg/ml per peptide), or PHA (10µg/ml). After incubation for 6 days in RPMI 1640 medium supplemented with 2 mM l-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM Hepes, and 10% heat-inactivated FCS, cells were washed, stained with viability dye, CD4 and CD8 antibodies, fixed and acquired on a LSR Fortessa flow cytometer. Suppression of T cell proliferation by Tregs was calculated as: (T cell proliferation (%) in the non-Treg co-culture – T cell proliferation (%) in the Treg co-culture)/T cell proliferation (%) in the non-Treg co-culture.

#### *Intracellular Cytokine Staining*

Purified CD3 T responder cells from HIV-1 patients were stained with CFSE as described above and subsequently mixed with sorted autologous Treg populations or control CD4 T cells at a ratio of 2:1. Cells were then stimulated with a pool of overlapping peptides spanning HIV-1 gag (2µg/ml per peptide) in the presence of antibodies directed against CD28 and CD49d (BD Biosciences, 2µg/ml).

Cells were incubated at 37°C for 6h, and brefeldin A (BD Biosciences) was added at 5µg/ml after the first hour of incubation. Afterwards, cells were stained with viability dye and antibodies against CD4 and CD8, fixed and permeabilized using a commercial kit (Caltag), and subjected to intracellular cytokine staining with monoclonal antibodies against interferon-γ (IFN-γ) and interleukin-2 (IL-2) (Becton Dickinson). Following final washes, cells were acquired on a LSR Fortessa instrument. The proportion of IFN-γ<sup>+</sup> or IL-2<sup>+</sup> CD4 and CD8 T cells was determined after gating on CD3<sup>+</sup> CFSE<sup>+</sup> responder T cells, using FlowJo software.

#### *Assessment of bystander activation*

Purified CD3<sup>+</sup> responder T cells from healthy individuals were mixed with sorted autologous Treg populations or autologous control CD4 T cells without regulatory activities at a ratio of 2:1. Following stimulation with Staphylococcal enterotoxin B (SEB, 5mg/ml, kindly provided by Dr. Eric J. Sundberg, University of Maryland), cells were incubated at 37°C for 4 days. Afterwards, cells were stained with antibodies against CD4, CD8, CD38, HLA-DR, and Vβ13.1 and viability dye before being subjected to flow cytometric acquisition on a LSR Fortessa instrument. The surface expression of activation markers in responder T cells was analyzed after gating on CD3<sup>+</sup> events. Treg-dependent suppression of bystander activation was calculated as: (CD38/HLA-DR-expressing T cells (%) in the non-Treg co-culture – CD38/HLA-DR-expressing T cells in the Treg co-culture)/CD38/HLA-DR-expressing T cells (%) in the non-Treg co-culture. For some experiments, T cells were stimulated by SEB for 4 days, and LILRB1 (clone HP-F1) expression was assessed on day 0, 1 and 4.

### *Virus production*

GFP-encoding X4-tropic NL4-3 and R5-tropic Ba-L HIV-1 plasmid, as well as HIV vector, were kindly provided by Drs. Nicholas Manel and Dan Littman, New York University, New York, NY (11).

Viral particles were produced by transfecting 293T cells with the respective HIV-1 plasmids (pCG-VSV-G was added for the single-round HIV vector), using TransIT-293 (Mirus) in OptiMEM following the manufacturer's instructions. Briefly, 293 T cells were seeded 24h prior to transfection in 10-cm plates. On the day of transfection, 45 µl TransIT – 293 reagent was added to 1ml OptiMEM in a sterile plastic tube, mixed and incubated at room temperature for 15 min. 15µg DNA was then added and incubated for another 30 min. Excess medium was removed from the plate and the TransIT-293/DNA mixture was added drop by drop to the cells. Gentle rocking of the dish was done to allow even distribution. Supernatants containing infectious retroviruses were harvested 48 hours after transfection, centrifuged, and stored at –80°C. Viral stock was treated with DNase I (30U/ml, Invitrogen) for 1 hour at room temperature before using.

### *Susceptibility assessment*

CD4 T cells were stimulated in RPMI 1640 medium supplemented with 2 mM l-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM Hepes, 10% heat-inactivated FCS, 50U/ml IL-2, and anti-CD3/CD8 bi-specific antibodies (0.5mg/ml). On day 5, cells were infected with X4 (MOI=0.02) or R5-tropic (MOI=0.07) viral strains for 4 h, or with VSV-G vector (MOI=0.02) for 2h at 37°C. After two washes, cells were resuspended in warm medium and plated at  $5 \times 10^5$  cells per well in 24-well plates. Cells were fed with fresh warm medium on day 2 for X4 and R5 virus.

On day 2 (VSV-G) or day 4 (X4, R5), harvested and stained the cells with antibodies against CD3, CD4, HLA-G, CCR5, CXCR4 and viability dye, and applied to a LSR Fortessa instrument.

For infection of quiescent cells, negatively-selected CD4 T cells with a purity of >98% were directly infected with HIV-1 vectors at MOI=0.01, X4 strain at MOI=0.005 and R5 strain at MOI=0.001; after *in vitro* culture for 96h in the absence of exogenous IL-2, cells were analyzed using the same staining panel by flow cytometric assessment.

#### *Statistical analysis*

Data are expressed as mean and standard deviation/standard error, or as box and whisker plots indicating the median, the 25% and 75% percentile and the minimum and maximum of all data. Differences between different cohorts or different experimental conditions were tested for statistical significance using Mann-Whitney U test, paired T test or one-way ANOVA, followed by post-hoc analysis using Tukey's multiple comparison test, as appropriate. Spearman correlation was used to assess the association between two variables. A p-value of 0.05 was considered significant.

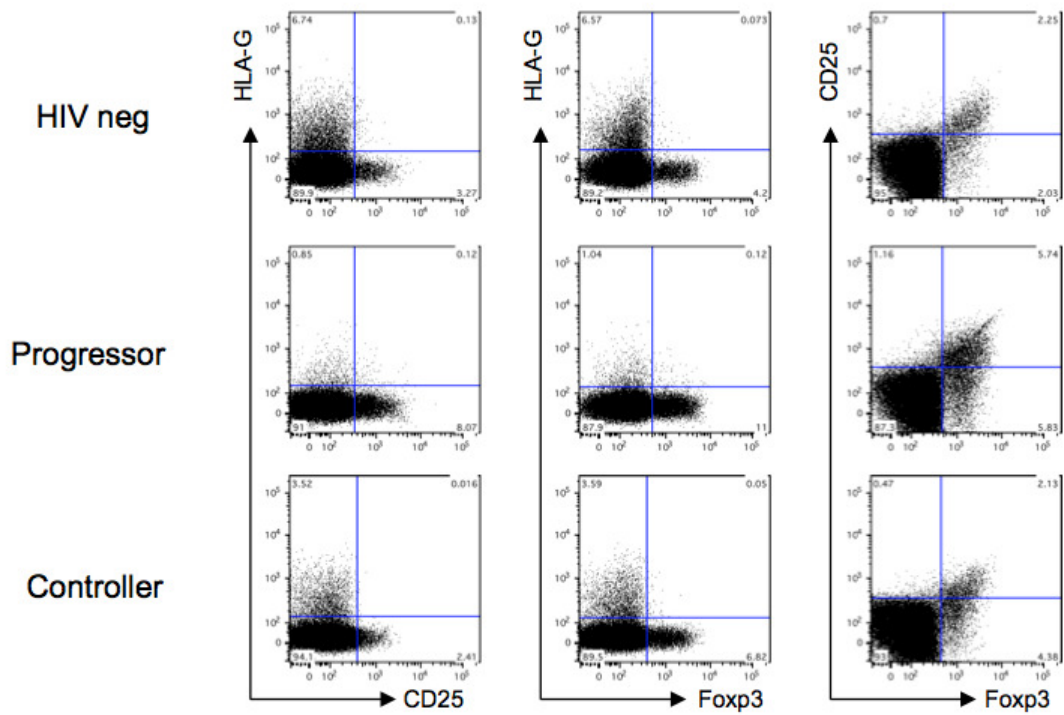
## Results

### *Reduced frequency of HLA-G<sup>+</sup> Treg cells in progressive HIV-1 infection*

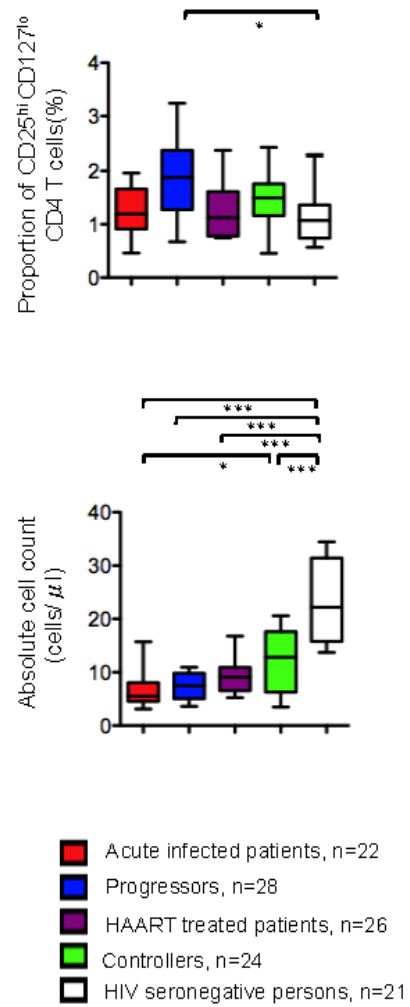
Investigations of T cells with regulatory properties in HIV-1 infection have so far been mostly limited to classical, CD25<sup>+</sup> and/or FoxP3 expressing Treg subset. To analyze the role of alternative, non-classical Treg populations in patients infected with HIV-1, we focused on the recently described population of Treg cells defined by surface expression of HLA-G (1). These cells do not express FoxP3 or CD25 (Figure 2-1), and are phenotypically and functionally distinct from classical Treg (1, 4). To analyze these cells in HIV-1 infection, we used flow cytometry to determine the relative and absolute numbers of HLA-G-positive CD4 and CD8 T cells in patients with primary HIV-1 infection and seroconversion within 3 months, treatment-naïve HIV-1 infected individuals with chronic progressive infection, HIV-1 infected persons successfully treated with HAART, as well as patients with spontaneous control of HIV-1 replication. A cohort of HIV-1 negative persons was recruited for control purposes.

Consistent with prior reports (12), we observed that relative proportions of classical CD25<sup>hi</sup> CD127<sup>low</sup> CD4 Treg were increased in progressive HIV-1 infection, while absolute Treg numbers were decreased (Figure 2-2); no correlations were found between frequency or absolute count of Treg cells and levels of immune activation (Figure 2-3).

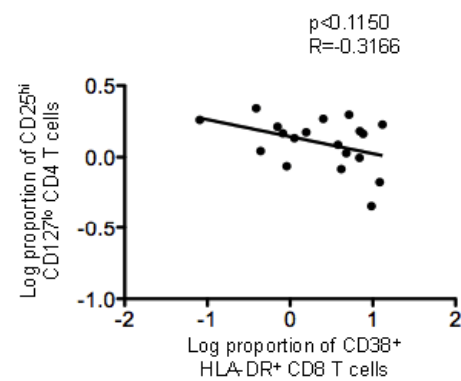
In contrast, we observed that the proportions and absolute numbers of HLA-G expressing CD4 T cells were lowest in HIV-1 progressors, while no significant differences were found between the proportions of HLA-G<sup>+</sup> CD4 T cells in any of the other HIV-1 patient cohorts (Figure 2-4, 2-5). The relative frequency of HLA-G expressing CD8 T cells was lower in all HIV-1 infected patient populations



**Figure 2-1.** Co-expression of HLA-G with CD25 and FoxP3 in patients with different HIV-1 stages and healthy individuals. Co-expression of CD25 with FoxP3 is shown as a control of the staining.

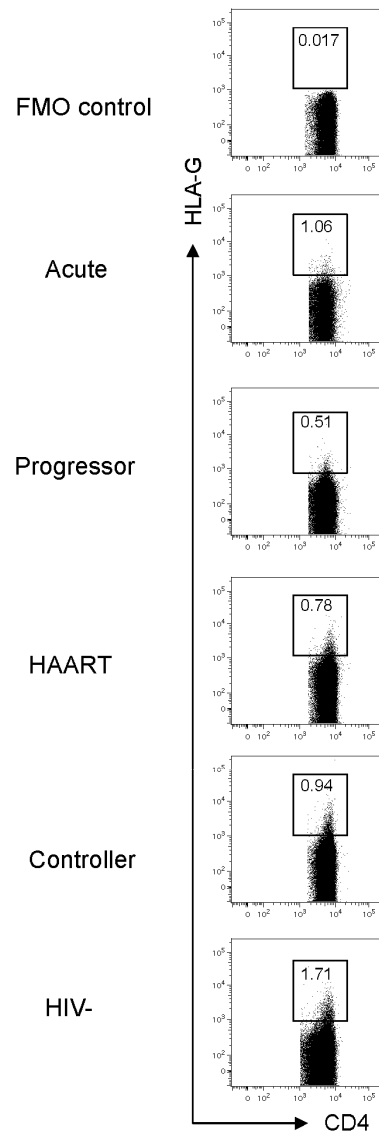


**Figure 2-2.** Relative proportions and absolute counts of classical CD25<sup>hi</sup>CD127<sup>lo</sup> CD4 Treg cells in indicated study populations. \*:p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

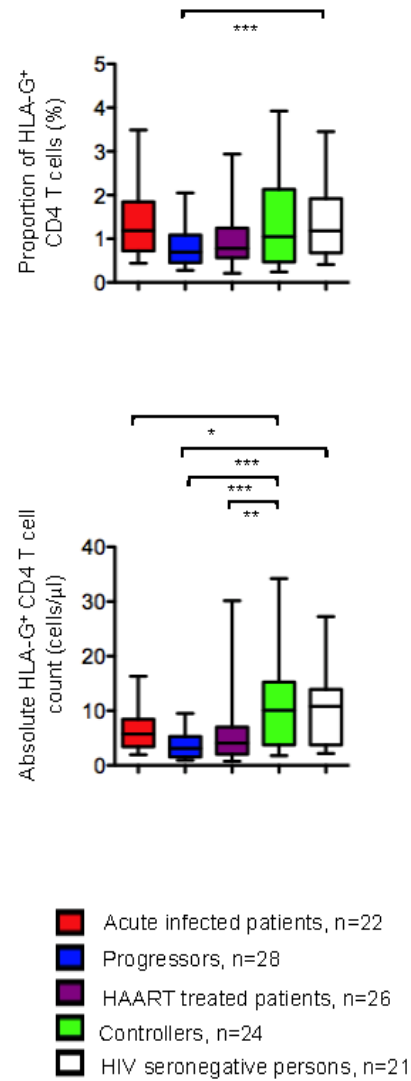


**Figure 2-3.** Correlation between CD25<sup>hi</sup> CD127<sup>lo</sup> CD4 T cell proportion and immune activation level, as indicated by CD38<sup>+</sup>HLA-DR<sup>+</sup> CD8 T cell percentage.





**Figure 2-4.** Diminished proportions of HLA-G<sup>+</sup> CD4 T cells in progressive HIV-1 infection. Representative flow cytometry dot plots reflect the proportion of HLA-G<sup>+</sup> CD4 T cells in indicated study subjects. FMO, Fluorescence minus one.

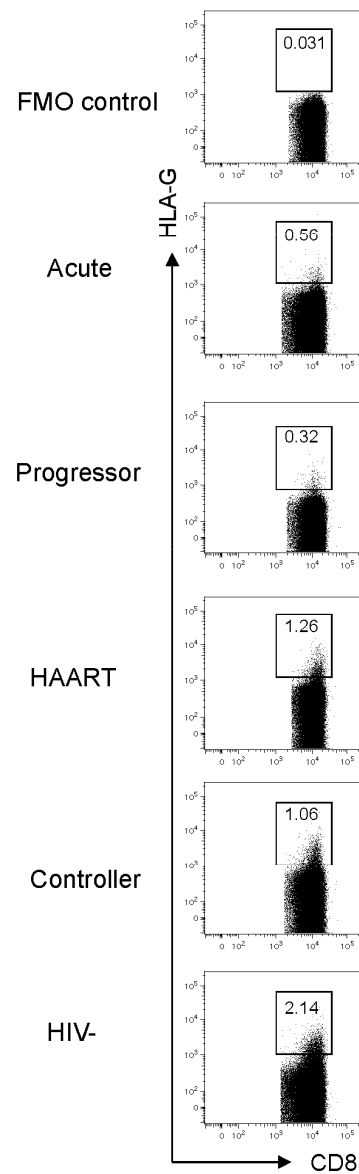


**Figure 2-5.** Diminished proportions of HLA-G<sup>+</sup> CD4 T cells in progressive HIV-1 infection. Box and Whisker plots summarize the frequencies and absolute counts of HLA-G<sup>+</sup> CD4 T cells in indicated study cohorts. \*:p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

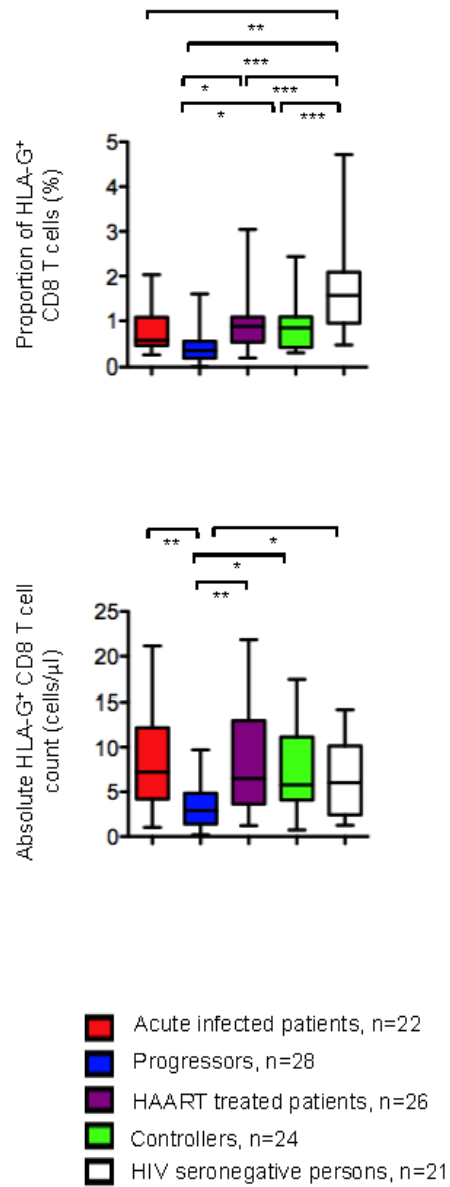
compared to HIV-1 negative persons; this reduction was again most pronounced in persons with untreated progressive disease (Figure 2-6, 2-7). We further stratified controllers by elite controllers (VL<50 copies/ml) and viremic controllers (50 copies/ml <VL< 2,000 copies/ml), and compared their HLA-G<sup>+</sup> Treg frequencies. In both CD4 and CD8 compartments, we observed a trend towards higher HLA-G<sup>+</sup> Treg proportions in elite controllers than viremic controllers (p=0.17 for CD4 and p=0.0636 for CD8, figure 2-8). Notably, the relative and absolute numbers of HLA-G expressing CD4 and CD8 T cells were positively correlated with CD4 T cell counts (Figure 2-9), a classic prognostic marker. Viral load in chronic progressors, however, does not correlate with HLA-G-expressing T cell proportions (Figure 2-10).

Interestingly, there was an inverse relationship between the frequency of HLA-G expressing T cells and corresponding levels of T cell immune activation, as determined by surface expression of HLA-DR and CD38 (Figure 2-11). Immune activation level is now widely recognized as a reliable prognostic marker of HIV-1 infection. Numerous groups have observed a strong correlation between immune activation level and HIV-1 disease progression, and indicated that the abnormal high immune activation was largely responsible for the loss of CD4 T cells (12-19). These correlations, therefore, strongly suggest an overall beneficial role of HLA-G-expressing Treg in HIV-1 infection.

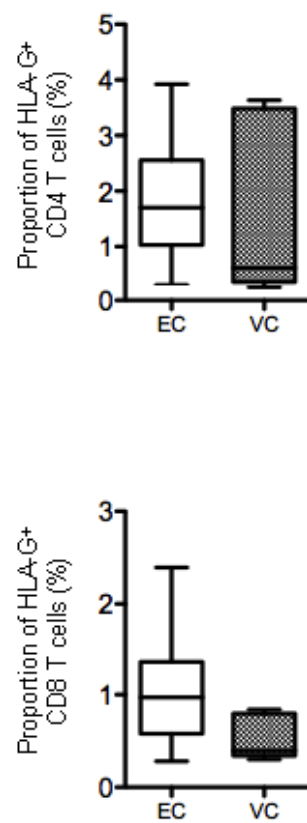
Taken together, these data indicate a selective numerical decrease of HLA-G expressing T cells in chronic progressive HIV-1 infection, and suggest that a reduction of HLA-G positive Tregs may contribute to higher levels of immune activation during progressive HIV-1 infection.



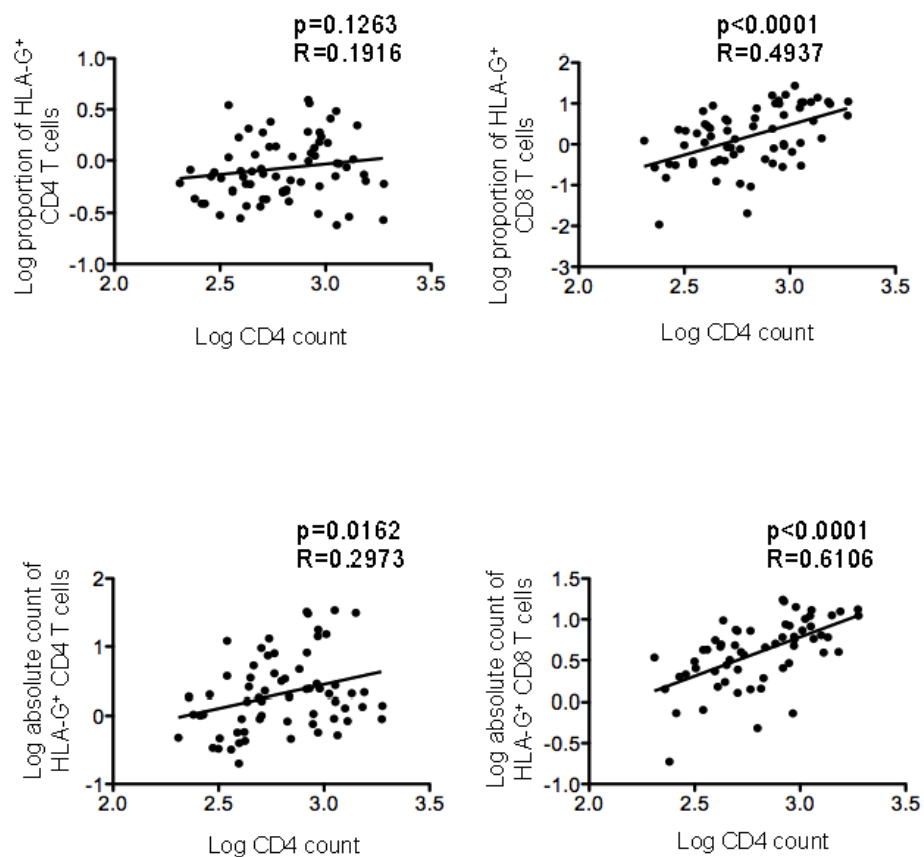
**Figure 2-6.** Diminished proportions of HLA-G<sup>+</sup> CD8 T cells in progressive HIV-1 infection. Representative flow cytometry dot plots reflect the proportion of HLA-G<sup>+</sup> CD8 T cells in indicated study subjects. FMO, Fluorescence minus one.



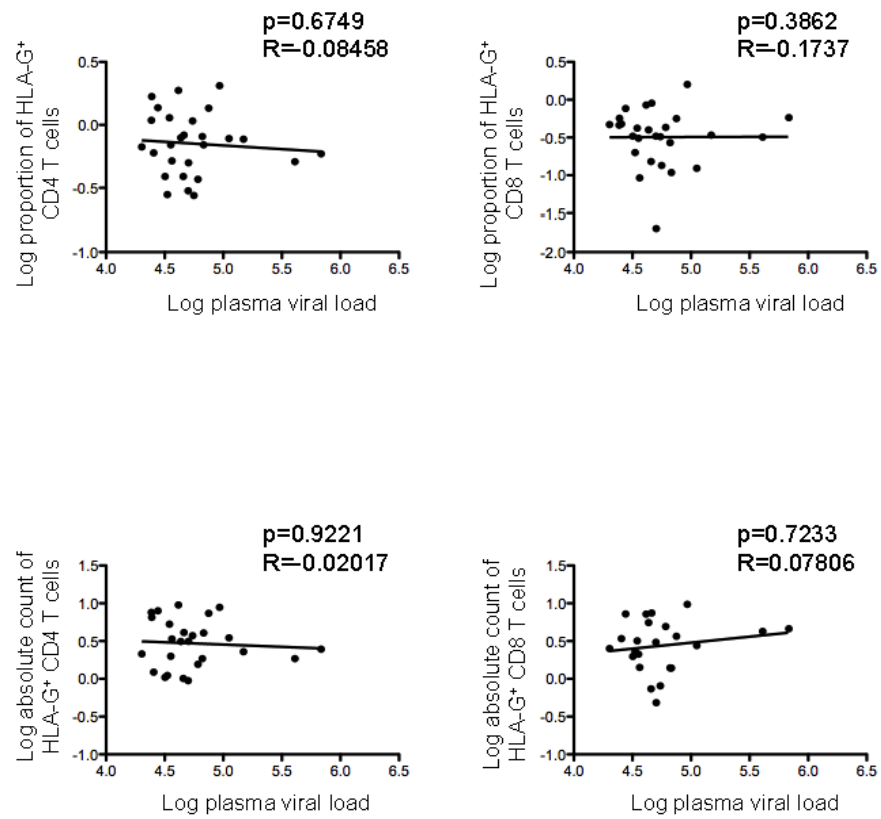
**Figure 2-7.** Diminished proportions of HLA-G<sup>+</sup> CD8 T cells in progressive HIV-1 infection. Box and Whisker plots summarize the frequencies and absolute counts of HLA-G<sup>+</sup> CD8 T cells in indicated study cohorts. \*:p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.



**Figure 2-8.** Proportions of HLA-G-expressing CD4 or CD8 T cells in elite controller (n=10) and in viremic controller (n=7).

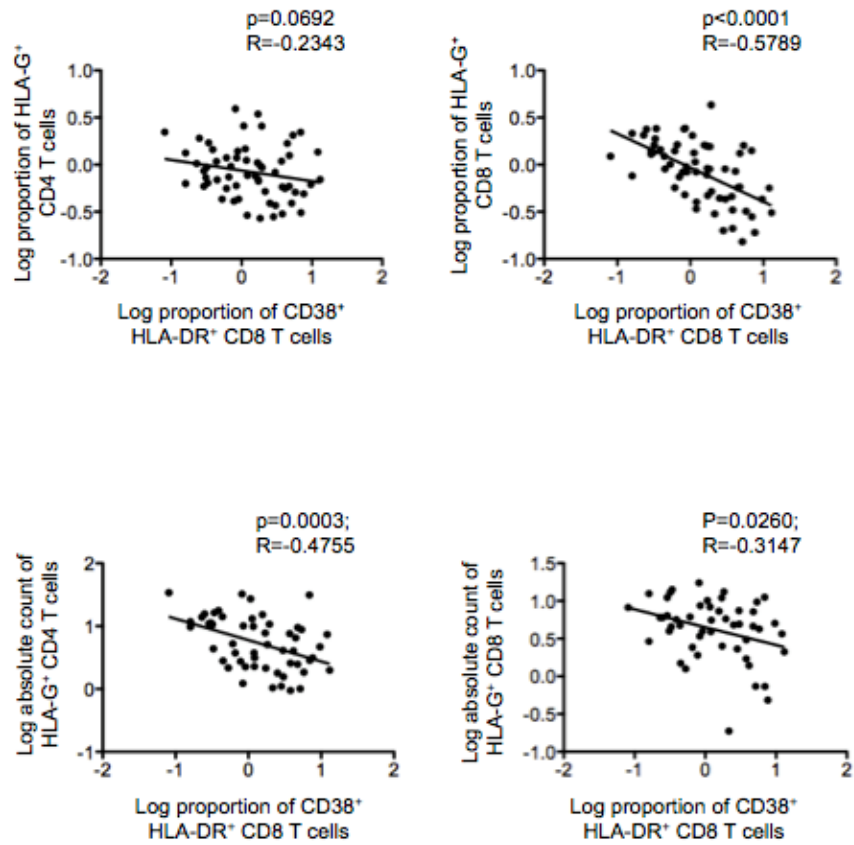


**Figure 2-9.** Correlations between HLA-G<sup>+</sup> T cell proportions and total CD4 T cell counts in controllers (n=23), progressors (n=27) and HIV seronegative individuals (n=15).



**Figure 2-10.** Correlations between HLA-G<sup>+</sup> T cell proportions and plasma viral load in progressors (n=27).





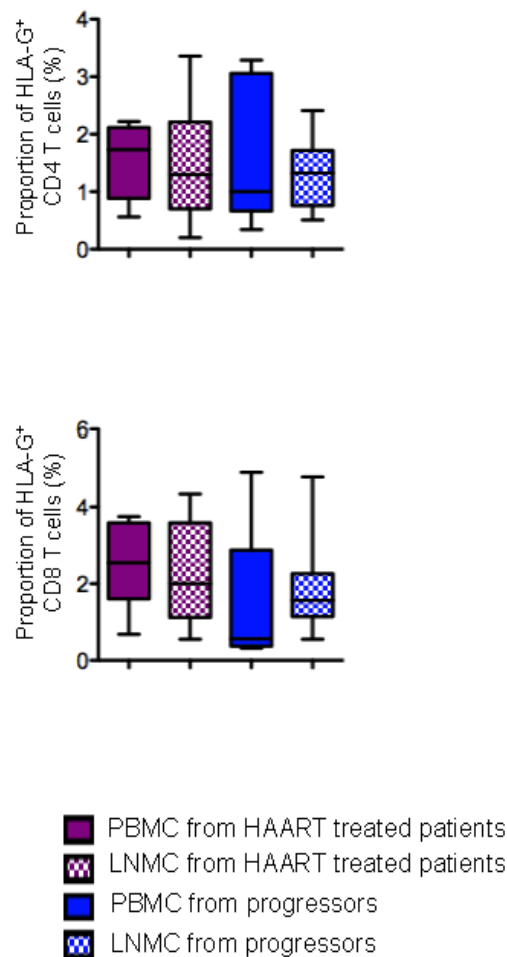
**Figure 2-11.** Correlations between HLA-G<sup>+</sup> T cell proportions and T cell immune activation. Immune activation level was determined by surface expression of CD38 and HLA-DR in controllers (n=19), progressors (n=20), and HIV seronegative individuals (n=15).

### *Depletion of peripheral HLA-G-expressing Treg cells is not likely due to tissue redistribution*

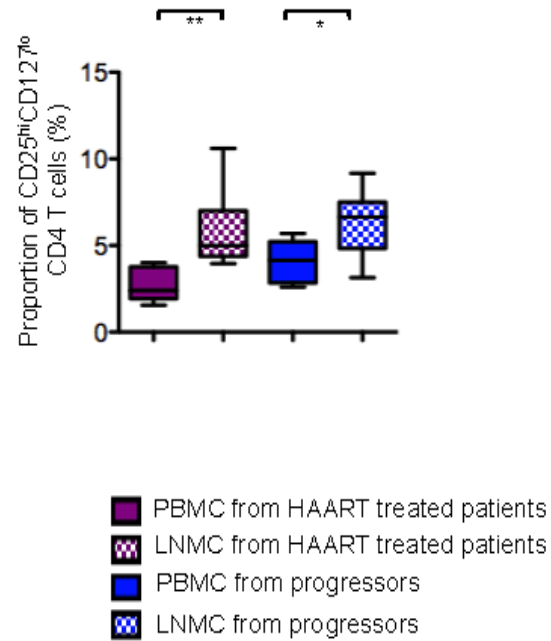
Since HLA-G<sup>+</sup> Treg cells express multiple tissue homing factors (20), a redistribution of these cells to lymphoid tissues may be responsible for the apparent reduction of HLA-G-expressing Treg cells in the peripheral blood during progressive HIV-1 infection. To investigate this, we analyzed the proportion of HLA-G<sup>+</sup> T cells in lymph node and peripheral blood samples collected from patients treated with antiretroviral therapy (HIV-1 viral load <75 copies/ml, median CD4 count: 762/μl [IQR 528-1,152]) or with untreated progressive HIV-1 infection (median HIV-1 viral load: 62,750 copies/ml [IQR 1,300-252,000], median CD4 count: 363/μl [IQR 254-610]). Among these patients, proportions of HLA-G<sup>+</sup> CD4 and CD8 Treg cells in lymph nodes and peripheral blood were not significantly different, suggesting that compartmentalization of HLA-G<sup>+</sup> Treg cells to lymph nodes does not represent the major reason explaining the decreased number of circulating HLA-G Treg cells in progressive HIV-1 infection (Figure 2-12). In contrast, classical CD25<sup>hi</sup>CD127<sup>lo</sup> Treg cells were significantly enriched in lymph nodes compared to peripheral blood in patients on and off HAART, consistent with previous results (Figure 2-13) (21).

### *Differentiation and maturation status of HLA-G<sup>+</sup> Treg cells*

We next investigated whether the different frequencies of HLA-G<sup>+</sup> CD4 and CD8 T cells during HIV-1 infection are associated with an altered phenotypic differentiation or maturation status of HLA-G<sup>+</sup> Treg cells. We found that in all study cohorts, the T cell subset distribution of HLA-G<sup>+</sup> CD4 T cells into naïve, central-memory, effector-memory and terminally-differentiated CD4 T cells was not



**Figure 2-12.** Proportion of HLA-G<sup>+</sup> CD4 and HLA-G<sup>+</sup> CD8 Treg cells in lymph node and peripheral blood. HAART: chronically-infected patients successfully treated with highly active antiretroviral therapy; Viremic: chronically-infected untreated patients. N=5 for PBMC samples from HAART-treated or progressors; n=12 for LNMC samples from HAART treated patients, and n=9 for LNMC samples from progressors.



**Figure 2-13.** CD25<sup>hi</sup>CD127<sup>lo</sup> CD4 Treg cells are enriched in lymph node compared to that in peripheral blood. HAART: chronically-infected patients successfully treated with highly active antiretroviral therapy; Viremic: chronically-infected untreated patients. N=5 for PBMC samples from HAART-treated or progressors; n=12 for LNMC samples from HAART treated patients, and n=9 for LNMC samples from progressors. \*:p<0.05; \*\*: p<0.01.

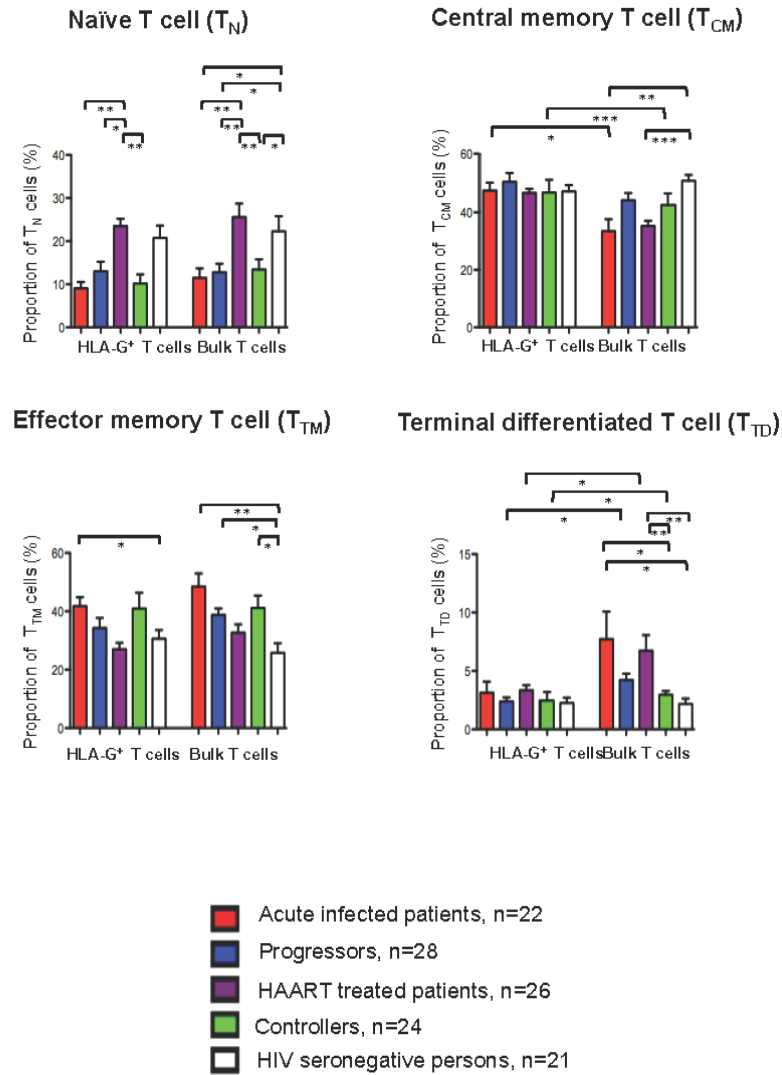
substantially different from corresponding bulk CD4 T cells, except a subtle skew of terminal differentiated subset to central memory population (Figure 2-14). Moreover, the expression of CD57 and PD-1, two surface markers associated with senescence and exhaustion of T cells, was not markedly different between HLA-G<sup>+</sup> CD4 T cells and the respective bulk CD4 T cells (Figure 2-15).

In contrast, we noted that in all study cohorts, HLA-G<sup>+</sup> CD8 T cells tended to have a more immature naïve or central-memory phenotype when compared to that of reference bulk CD8 cell populations (Figure 2-16). There was also a trend toward reduced surface expression of CD57 surface expression on HLA-G<sup>+</sup> CD8 T cells in comparison to that on corresponding bulk CD8 T cells (Figure 2-17).

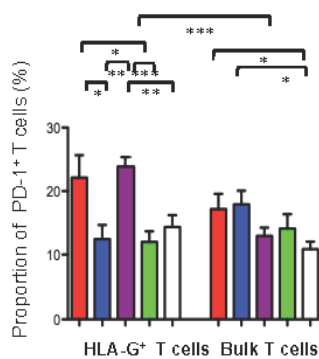
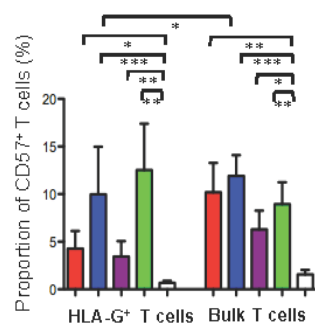
Overall, these data indicate that during HIV-1 infection, HLA-G expressing CD8, but not CD4 T cells, are skewed toward a more immature differentiation status, but this difference is not correlated to the rates of spontaneous HIV-1 disease progression.

#### *HLA-G-expressing Treg cells do not inhibit functional properties of HIV-1-specific T cells*

A functional hallmark of classical Treg cells is their ability to inhibit antigen-specific T cell responses (22). Prior work has shown that non-classical Treg cells can also inhibit proliferative properties of T cells, but their functional effects on HIV-1-specific, or other antigen-specific T cells remain unclear (1). To investigate this, CFSE-labeled PBMCs from HIV-1 patients were stimulated with a pool of overlapping HIV-1 Gag peptides, CMV pp65 peptide pool, or PHA. The stimulated cells were then individually mixed with sorted autologous HLA-G<sup>+</sup> CD4 T cells, HLA-G<sup>+</sup> CD8 T cells, or HLA-G<sup>-</sup> CD25<sup>+</sup> CD4 T cells; HLA-G<sup>-</sup> CD25<sup>-</sup> CD4 or HLA-G<sup>-</sup> CD25<sup>-</sup>

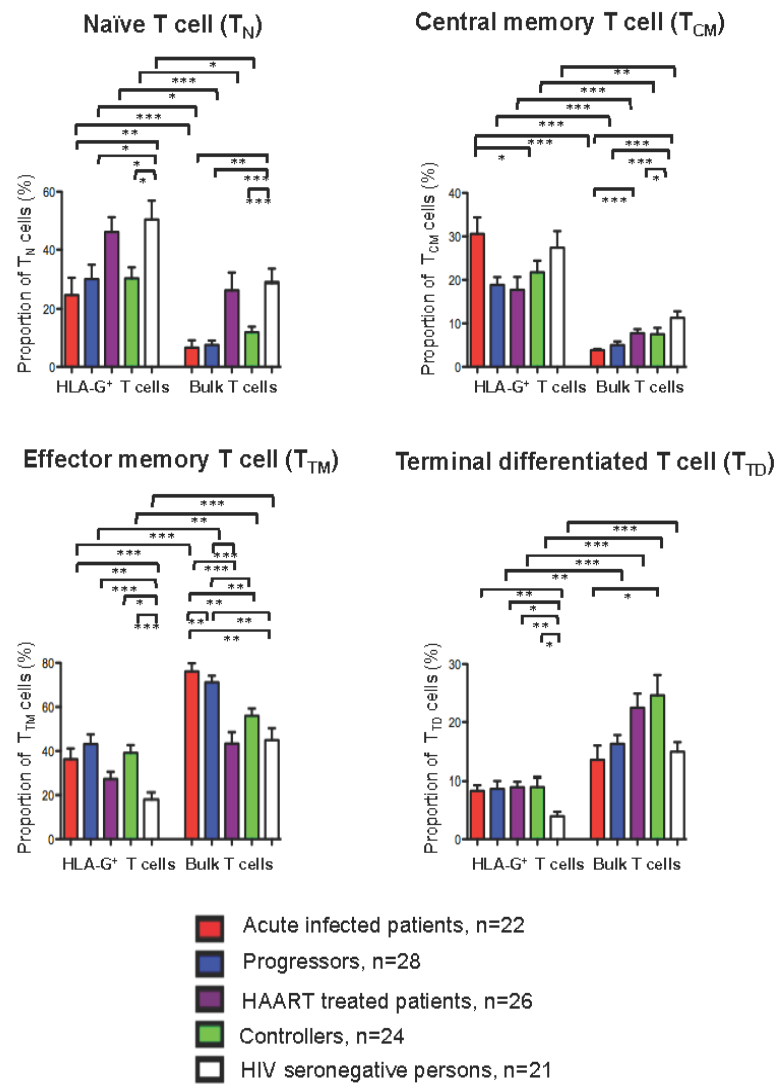


**Figure 2-14.** T cell subset distribution of HLA-G-expressing CD4 T cells in indicated study populations. Data from corresponding bulk T cell populations are shown for reference purposes. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .



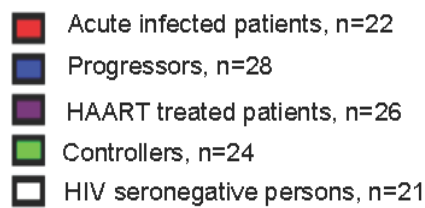
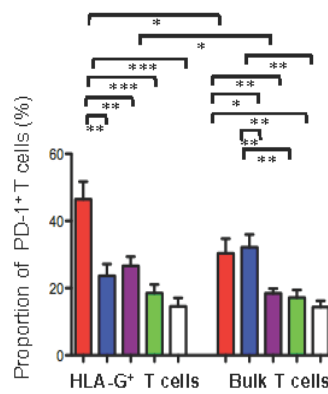
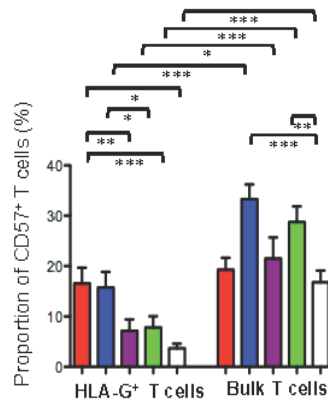
- Acute infected patients, n=22
- Progressors, n=28
- HAART treated patients, n=26
- Controllers, n=24
- HIV seronegative persons, n=21

**Figure 2-15.** Surface expression of CD57 and PD-1 in HLA-G- expressing CD4 T cells in indicated study cohorts. Data from corresponding bulk T cell populations are shown for reference purposes. \*:p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.



**Figure 2-16.** T cell subset distribution of HLA-G-expressing CD8 T cells in indicated study populations. Data from corresponding bulk T cell populations are shown for reference purposes. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .





**Figure 2-17.** Surface expression of CD57 and PD-1 in HLA-G<sup>+</sup> expressing CD8 T cells in indicated study cohorts. Data from corresponding bulk T cell populations are shown for reference purposes. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

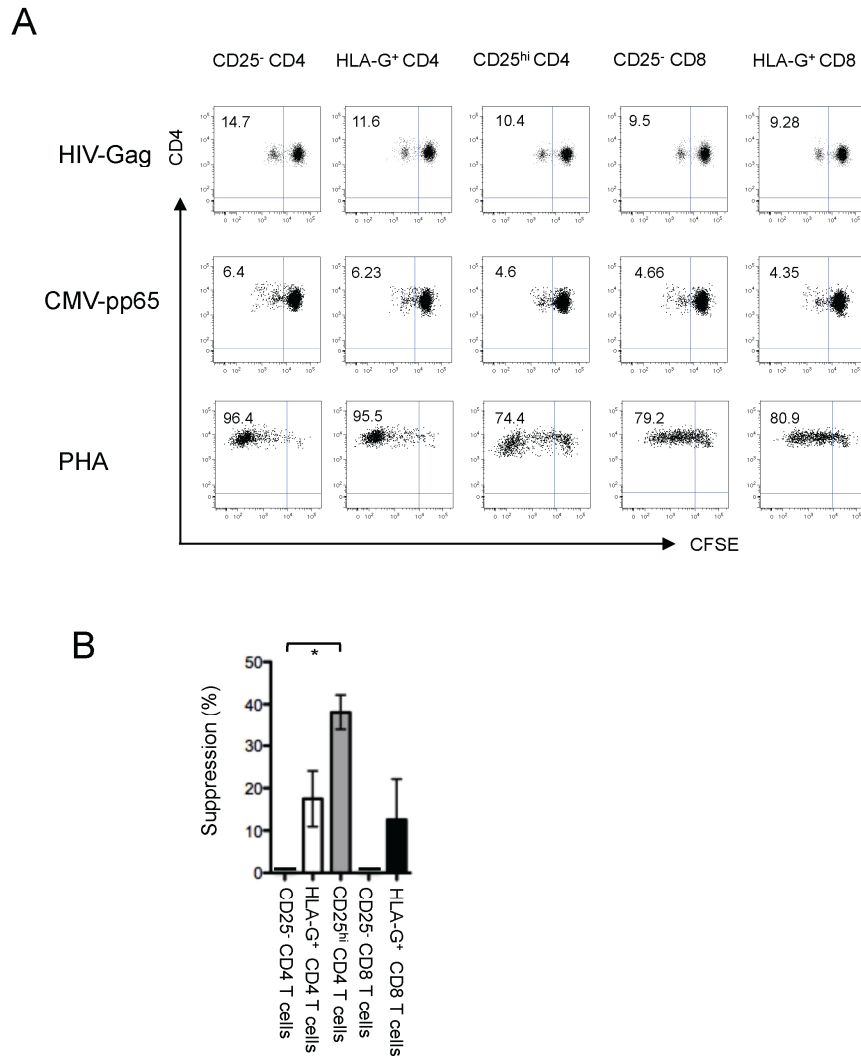
CD8 conventional T cells were added as a negative control. Subsequently, proliferation of antigen-specific CD4 and CD8 T cells was monitored after six days of culture.

These experiments demonstrated relatively strong suppressive effects of classical CD25<sup>+</sup>CD4<sup>+</sup> Treg on the proliferative activities of HIV-1-specific cells, consistent with prior reports showing potent Treg-mediated inhibition of CD8 T cell proliferation (23). HLA-G-expressing Tregs, however, did not significantly reduce the proliferation of HIV-1-specific CD4 and CD8 T cells (Figure 2-18, 2-19).

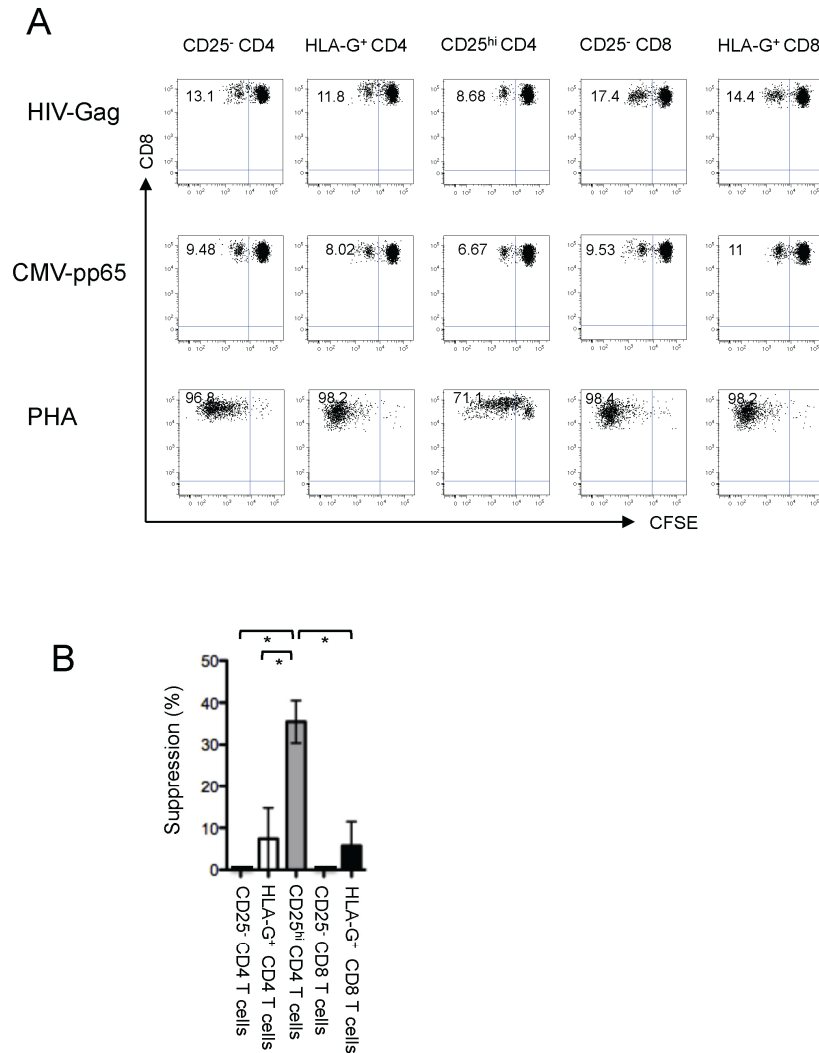
To assess whether HLA-G-expressing Tregs influence the cytokine secretion properties of HIV-1-specific T cells, isolated CD3<sup>+</sup> T cells from HIV-1-infected persons were stimulated with overlapping Gag peptides in the presence of the HLA-G<sup>+</sup> CD4<sup>+</sup> T cells, HLA-G<sup>-</sup> CD25<sup>+</sup> CD4<sup>+</sup> T cells, or HLA-G<sup>-</sup> CD25<sup>-</sup> control cells; after 16 hours of incubation, antigen-specific secretion of interferon- $\gamma$  and IL-2 was monitored by flow cytometry. As summarized in Figure 2-20, cytokine secretion of HIV-1-specific CD8 T cells did not differ following co-incubation with any of the Treg populations or negative control cells. Taken together, these data show that HLA-G-expressing Tregs have limited effects on the functional activities of HIV-1-specific T cells, at least under the tested conditions.

#### *HLA-G expressing Tregs selectively inhibit bystander activation*

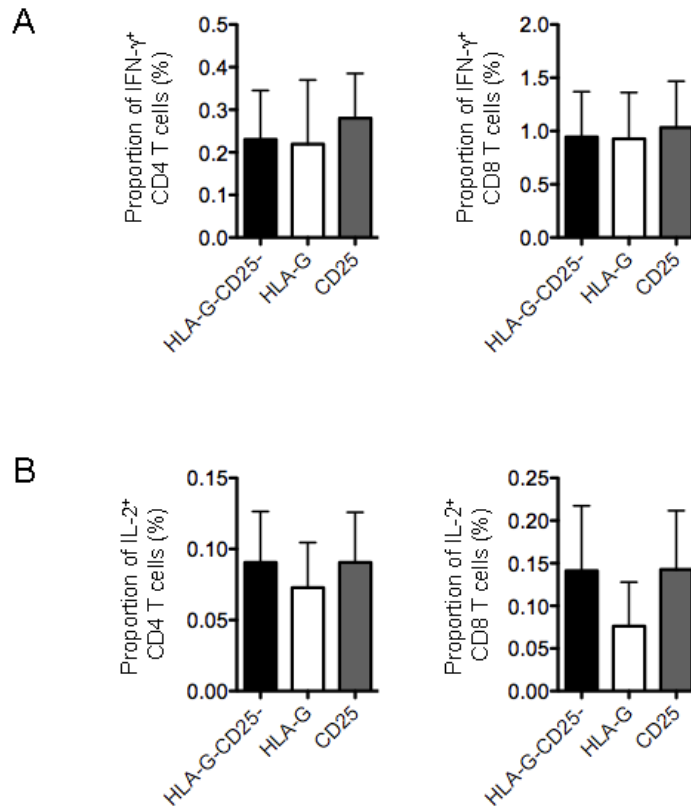
To further explore the role of HLA-G-expressing Treg cells in HIV-1 disease pathogenesis, we focused on how they influence T cell activation. Activation of T lymphocytes can either occur through direct antigenic triggering of the TCR, or by mechanisms involving a TCR-independent mode of T cell stimulation, commonly referred to as “bystander activation” (24, 25). Both of these pathways seem to



**Figure 2-18.** HLA-G-expressing Treg cells do not inhibit proliferative activities of HIV-1, CMV-, or PHA-stimulated helper T cells. (A): Representative dot plots reflect proliferative activities of HIV-Gag, CMV-pp65, or PHA-stimulated CD4 T cells following incubation with indicated autologous Treg subsets or HLA-G<sup>-</sup> CD25<sup>-</sup> control cells (B): Cumulative data from n=3 study subjects indicate Treg mediated suppressive effects, if any, on CD4 T cell proliferation. \*:p<0.05.



**Figure 2-19.** HLA-G-expressing Treg cells do not inhibit proliferative activities of HIV-1, CMV-, or PHA-stimulated cytotoxic T cells. (A): Representative dot plots reflect proliferative activities of HIV-gag, CMV-pp65, or PHA-stimulated CD8 T cells following incubation with indicated autologous Treg subsets or HLA-G<sup>+</sup> CD25<sup>-</sup> control cells. (B): Cumulative data from n=3 study subjects indicate the Treg mediated suppressive effects, if any, on CD8 T cell proliferation. \*:p<0.05.



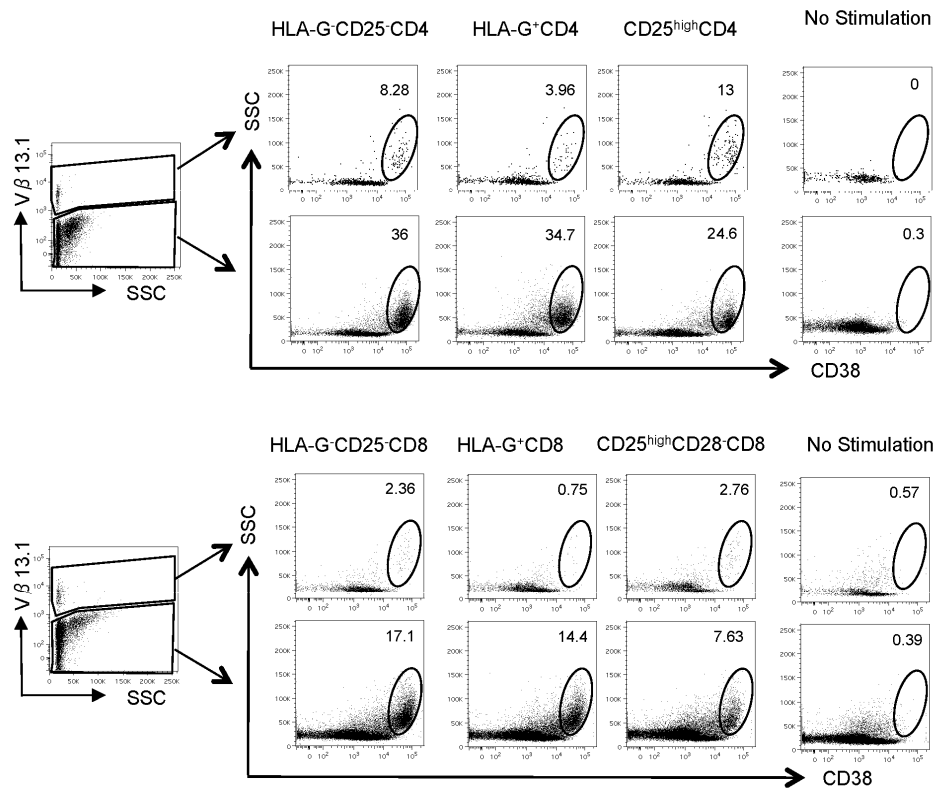
**Figure 2-20.** HLA-G<sup>+</sup> Treg cells do not affect cytokine secretion properties of HIV-1-specific T cells. (A): Cumulative data from n=5 study subjects indicating the proportion of IFN<sup>+</sup> CD4 and CD8 T cells after co-incubation with indicated Treg subsets or control CD4 T cells. (B): Cumulative data from n=5 study subjects indicating the proportion of IL-2<sup>+</sup> CD4 and CD8 T cells after co-incubation with indicated Treg subsets or control CD4 T cells.

contribute to the pathological immune activation observed during progressive HIV-1 infection (18, 26), and may be influenced by the HLA-G<sup>+</sup> Treg populations described in this manuscript.

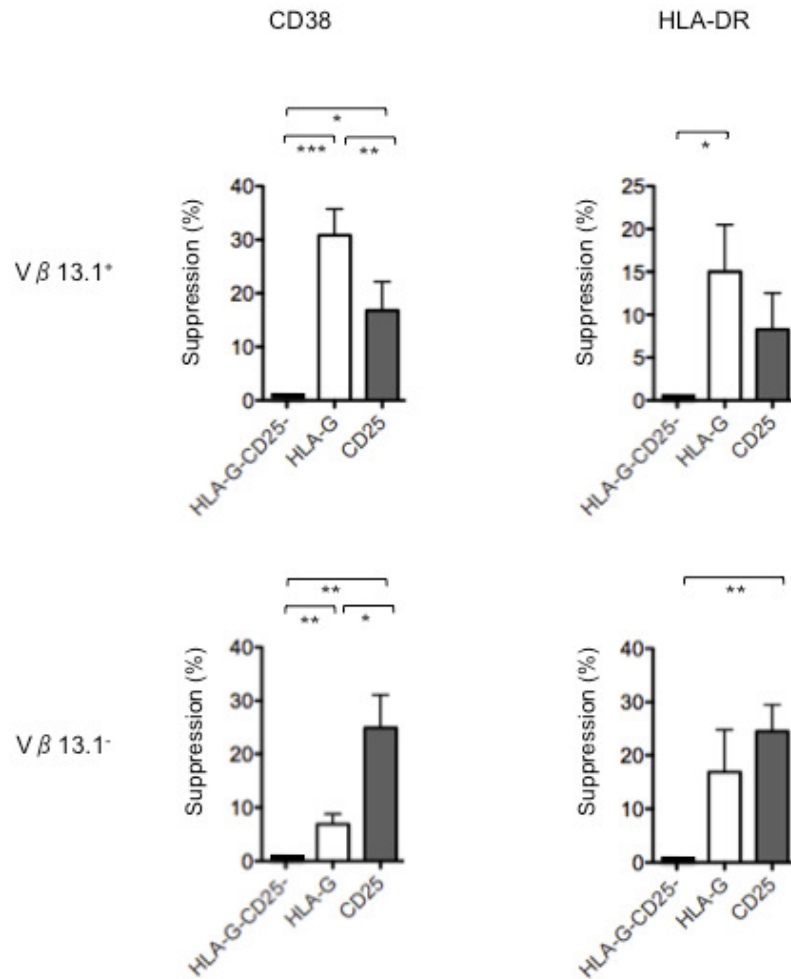
As a functional assay to investigate and quantify the effects of HLA-G<sup>+</sup> Treg cells on TCR-dependent and bystander immune activation, we stimulated T cells with staphylococcal enterotoxin B (SEB), an antigen that elicits T cell responses by a broad panel of different TCR clonotypes, but cannot be recognized by T cells using TCR V $\beta$ 13.1 (27, 28). Immune activation in V $\beta$ 13.1-expressing T cells following exposure to SEB can therefore only be attributed to bystander activation, while immune activation in V $\beta$ 13.1-negative T cells after SEB exposure mainly reflects classical TCR-dependent activation.

To analyze the effects of non-classical Tregs on immune activation, SEB-stimulated responder T cells were individually co-cultured with autologous populations of sorted HLA-G<sup>+</sup> CD4 Treg and CD25<sup>+</sup> CD4 Treg cells; HLA-G<sup>-</sup> CD25<sup>-</sup> CD4 were added for control purposes. Alternatively, HLA-G<sup>+</sup> CD8 Treg, CD25<sup>+</sup> CD28<sup>-</sup> CD8 Treg or HLA-G<sup>-</sup> CD25<sup>-</sup> CD8 control cells were added to autologous SEB-stimulated responder T cells. On day 4 of culture, immune activation was measured by flow cytometric analysis of CD38 and HLA-DR surface expression in V $\beta$ 13.1-positive and V $\beta$ 13.1-negative T cells.

As demonstrated in Figure 2-21, we observed that classical CD25<sup>+</sup> Treg cells potently suppressed CD38/HLA-DR expression in V $\beta$ 13.1-negative T cells, consistent with prior reports about the immunosuppressive properties these cells (23). In contrast, HLA-G expressing T cells led to a significantly reduced surface expression of HLA-DR and CD38 on V $\beta$ 13.1-expressing T cells, but had limited effects on immune activation of V $\beta$ 13.1-negative cells (Figure 2-22, 2-23). This

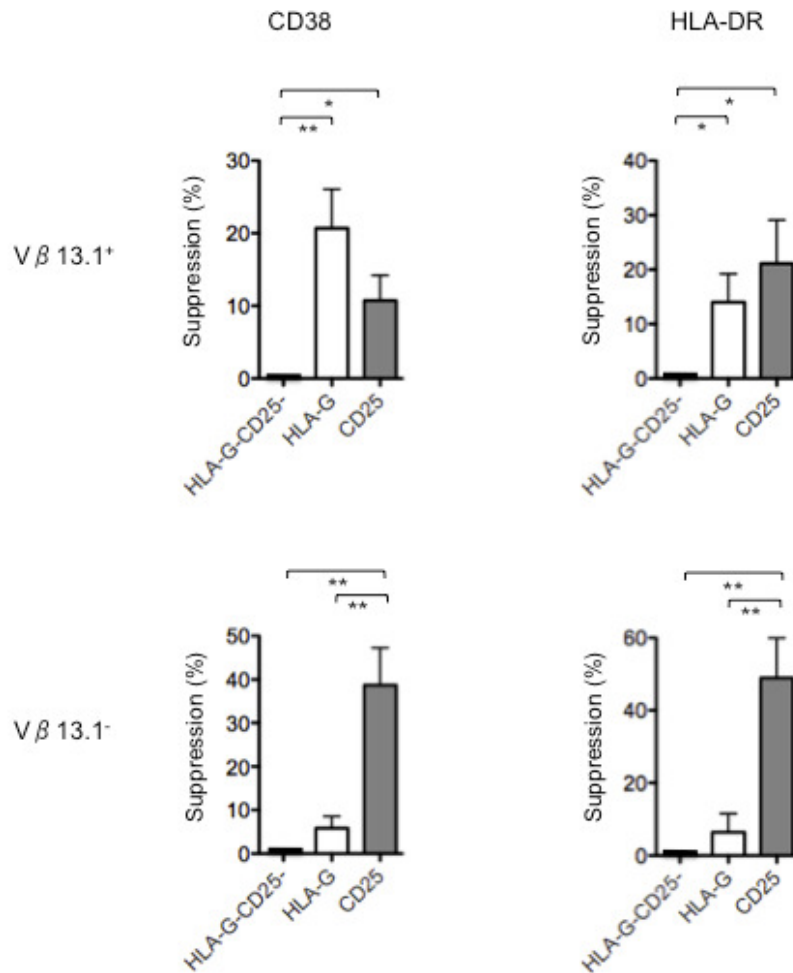


**Figure 2-21.** Representative flow cytometry dot plots reflect CD38 expression on Vβ13.1<sup>+</sup> and Vβ13.1<sup>-</sup> responder T cells. Responder cells were isolated from HIV seronegative individuals and activated by SEB in the presence of autologous Treg or conventional T cells.



**Figure 2-22.** Cumulative data represents relative suppression of CD38 and HLA-DR expression on Vβ13.1<sup>+</sup> and Vβ13.1<sup>-</sup> responder T cells following exposure to regulatory or conventional CD4 T cells. Responder cells were isolated from HIV seronegative individuals and activated by SEB, in the presence of autologous CD4 Treg or conventional, non-suppressive CD4 T cells. Data from n=8 study subjects are shown. \*:p<0.05; \*\*:p<0.01; \*\*\*: p<0.001.





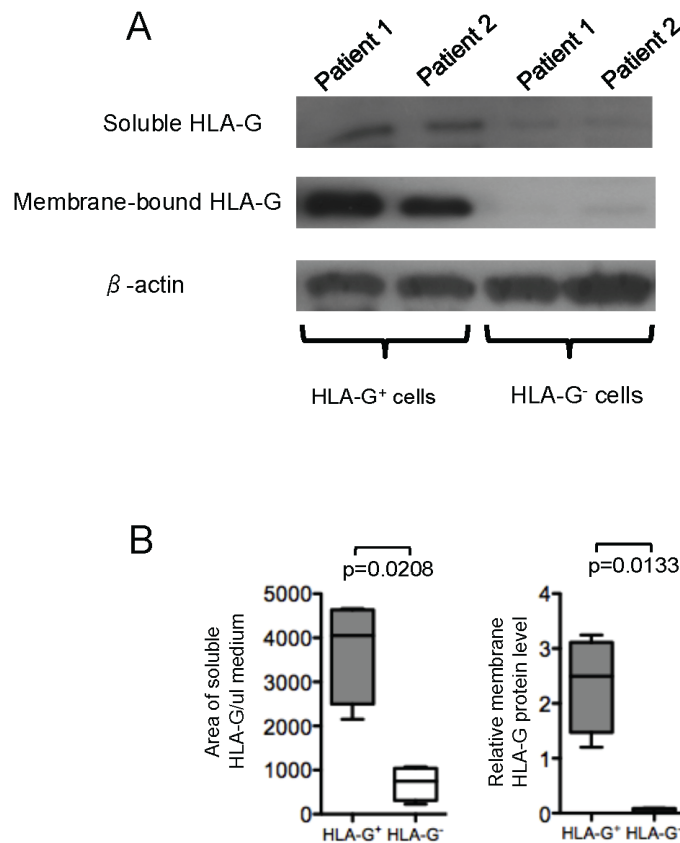
**Figure 2-23.** Cumulative data represents relative suppression of CD38 and HLA-DR expression on Vβ13.1<sup>+</sup> and Vβ13.1<sup>-</sup> responder T cells following exposure to regulatory or conventional CD8 T cells. Responder cells were isolated from HIV seronegative individuals and activated by SEB, in the presence of autologous CD8 Treg or conventional, non-suppressive CD8 T cells. Data from n=8 study subjects are shown. \*:p<0.05; \*\*: p<0.01.

selective inhibitory effect on bystander activation was seen both for HLA-G<sup>+</sup> CD4 and CD8 T cells and substantially exceeded regulatory effects on bystander activation of classical CD25<sup>+</sup> Treg.

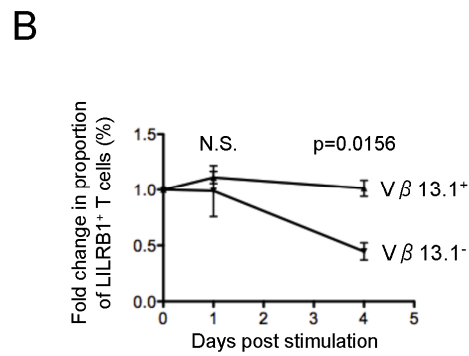
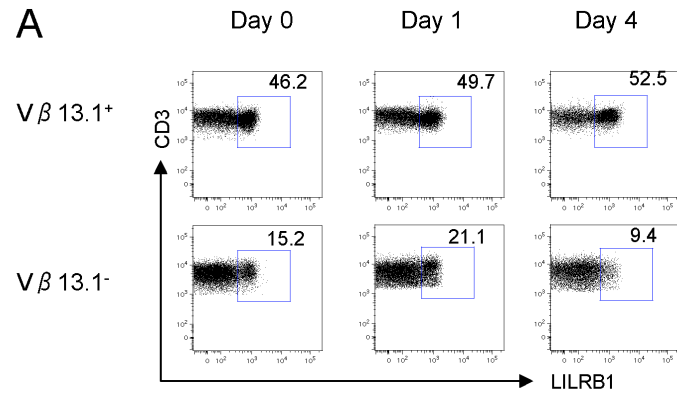
To explore reasons for the differential susceptibility of Vβ13.1-positive and Vβ13.1-negative responder T cells to classical and non-classical Treg, we analyzed the dynamics of LILRB1 surface expression on responder T cell over a 4-day incubation period. LILRB1 can effectively inhibit functional properties of T cells (29) and represents one of the highest-affinity receptors for HLA-G (30), which is secreted by HLA-G<sup>+</sup> Treg cells (Figure 2-24), and responsible for the immunomodulatory effects of these cells (1). Interestingly, we observed that following TCR-dependent T cell activation, LILRB1 surface expression on responder T cells declined, while that on bystander subsets was stable or slightly increased (Figure 2-25). We also measured the expression of another HLA-G receptor, KIR2DL4, that is primarily expressed on NK cells and certain T cell lines (31, 32). However we did not observe KIR2DL4 expression on primary T cells in our experimental system (data not shown). Overall, these data indicate that HLA-G<sup>+</sup> Treg cells differ from alternative Treg populations by their ability to potentially suppress bystander activation of T cells, and suggest that TCR-dependent and TCR-independent mechanisms of immune activation are associated with altered susceptibilities to inhibitory effects of classical and non-classical Treg cells.

#### *HLA-G-expressing CD4 T cells are more susceptible to HIV-1 infection*

Conventional CD25<sup>+</sup>CD4 Treg cells express HIV-1 co-receptors and are targets for HIV-1 infection (33, 34). Direct HIV-1 infection of HLA-G<sup>+</sup> CD4 Treg cells may contribute to the reduction of these cells in progressive HIV-1 infection. To

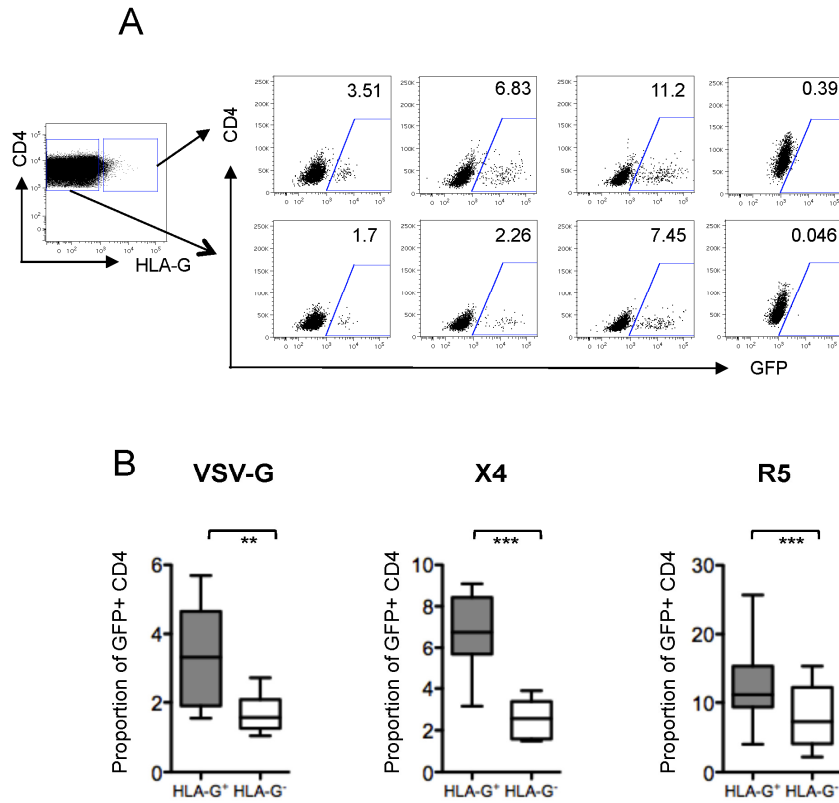


**Figure 2-24.** Expression of soluble and membrane-bound HLA-G in culture. (A): Western blots reflect soluble HLA-G level, cell-associated HLA-G and  $\beta$ -actin protein expression in isolated HLA-G<sup>+</sup> and HLA-G<sup>-</sup> subsets (B): Quantitative expression of soluble HLA-G and cell-associated protein. HLA-G<sup>+</sup> cells were pulled down by staining with a primary PE antibody followed by anti-PE beads. HLA-G<sup>-</sup> cells were collected in the flow through. Aliquot the same number of HLA-G<sup>+</sup> and HLA-G<sup>-</sup> cells in FACS tubes with R10/50 as the culture medium. Collect cells and supernatant for western blot analysis on Day 4. N=4.

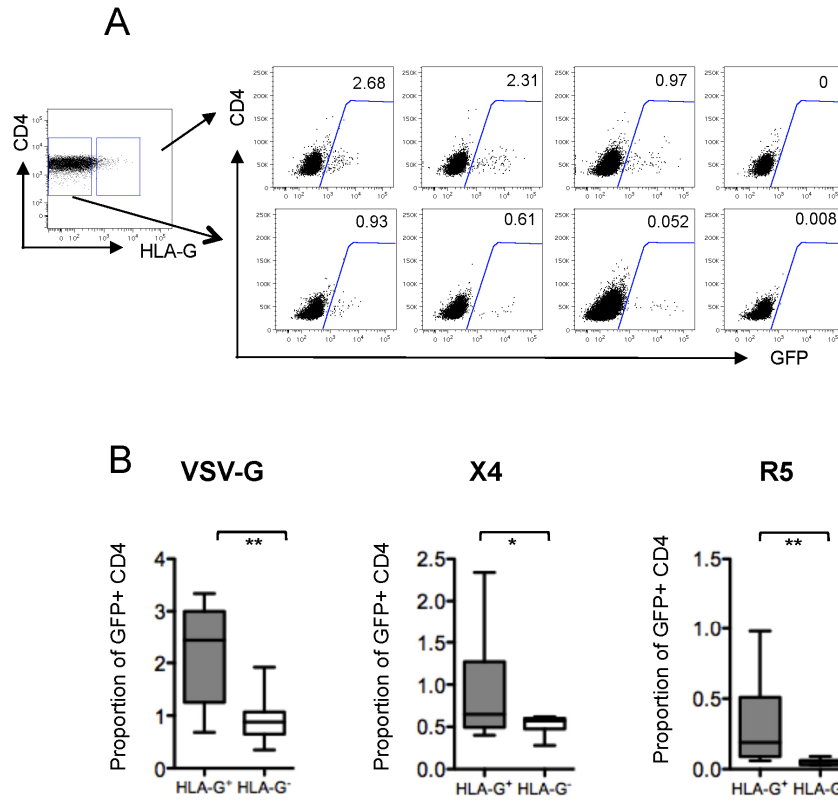


**Figure 2-25.** LILRB1 expression on  $V\beta 13.1^+$  T cells remain stable while that on  $V\beta 13.1^-$  T cells decreases. Representative plots (A) and statistic analysis (B) show the fold change of LILRB1 expression on  $V\beta 13.1^+$  and  $V\beta 13.1^-$  T cells during 4 days in culture. N=7.

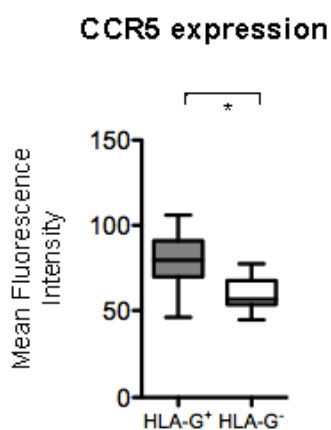
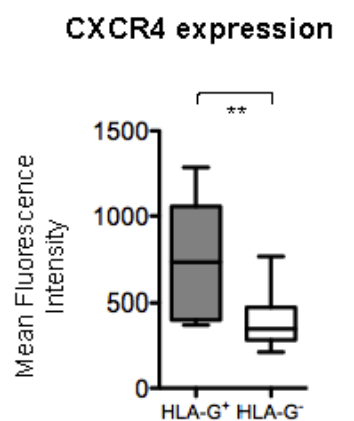
investigate this, we infected pre-activated or non-pre-activated (directly isolated *ex vivo*) CD4 T cells with X4- or R5-tropic virus, or with VSV-G pseudotyped construct that causes single round HIV-1 infection. After 4 days in culture, we observed that HLA-G<sup>+</sup> CD4 Treg were significantly more susceptible to HIV-1 infection than autologous HLA-G-negative CD4 T cells; this was true both for *in vitro* activated cells, and for cells directly infected *ex-vivo* (Figure 2-26, 2-27). This enhanced susceptibility was in line with higher expression of the HIV-1 co-receptors CXCR4 and CCR5 on HLA-G<sup>+</sup> CD4 Treg cells, in comparison to HLA-G-negative CD4 T cells (Figure 2-28 and 2-29). These data suggest that reduction of circulating HLA-G<sup>+</sup> Treg in progressive HIV-1 infection may, at least in part, be due to their enhanced susceptibility to HIV-1 infection.



**Figure 2-26.** HLA-G<sup>+</sup> Treg cells were more susceptible to VSV-G, X4 and R5 virus infection than the HLA-G<sup>-</sup> cells (with pre-activation). (A) Representative flow plots reflect the proportion of GFP-expressing HLA-G<sup>+</sup> or HLA-G<sup>-</sup> CD4 T cells. (B) Box and Whisker plots summarize the proportion of GFP-positive CD4 T cells in HLA-G<sup>+</sup> or HLA-G<sup>-</sup> cells. N=10. \*\*: p<0.01; \*\*\*: p<0.001.

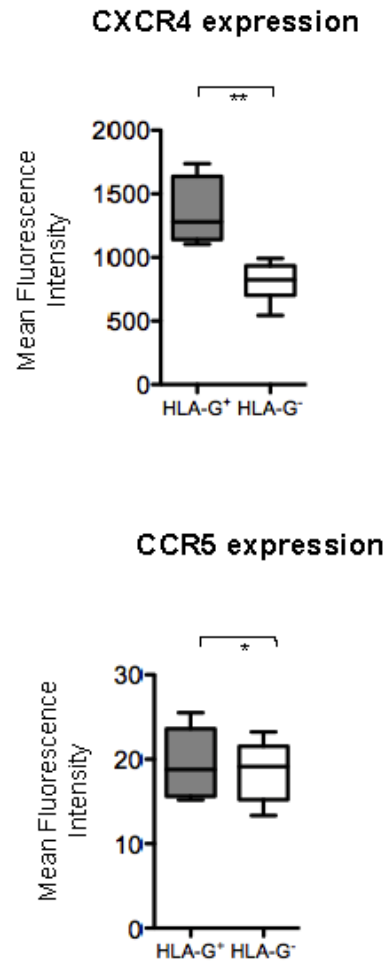


**Figure 2-27.** HLA-G<sup>+</sup> Treg cells were more susceptible to VSV-G, X4 and R5 virus infection than the HLA-G<sup>-</sup> cells (without pre-activation). (A) Representative flow plots reflect the proportion of GFP-expressing HLA-G<sup>+</sup> or HLA-G<sup>-</sup> CD4 T cells. (B) Box and Whisker plots summarize the proportion of GFP-positive CD4 T cells in the HLA-G<sup>+</sup> or HLA-G<sup>-</sup> cells. N=9. \*:p<0.05; \*\*:p<0.01.



**Figure 2-28.** Pre-activated HLA-G<sup>+</sup> CD4 T cells express higher level of HIV-1 co-receptors, CXCR4 and CCR5, than that of HLA-G<sup>-</sup> cells during *in vitro* infection. Box and Whisker plots reflect mean fluorescence intensity of CXCR4 and CCR5 in HLA-G<sup>+</sup> or HLA-G<sup>-</sup> cells. N=10. \*:p<0.05; \*\*: p<0.01.





**Figure 2-29.** Non-activated HLA-G<sup>+</sup> CD4 T cells express higher level of HIV-1 co-receptors, CXCR4 and CCR5, than that of HLA-G<sup>-</sup> cells during *in vitro* infection. Box and Whisker plots reflect mean fluorescence intensity of CXCR4 and CCR5 in HLA-G<sup>+</sup> or HLA-G<sup>-</sup> cells. N=9. \*:p<0.05; \*\*: p<0.01.

## Discussion

Regulatory T lymphocytes can influence immune homeostasis by suppressing innate and adaptive effector cell activity, and in this way may importantly modulate immune defense mechanisms against HIV-1 (35). The majority of currently available data indicate that classical CD25<sup>hi</sup>CD127<sup>lo</sup> Treg cells are expanded during chronic progressive HIV-1 infection (36-41) and may worsen spontaneous HIV-1 disease progression by potentially suppressing functional activities of HIV-1-specific T cell responses (23). Here, we demonstrate several numerical and functional aspects of non-classical HLA-G expressing Treg cells in HIV-1 infection that clearly distinguish them from these recognized characteristics of classical Treg subsets. We found that absolute numbers and relative proportions of HLA-G expressing Treg cells are diminished in progressive HIV-1 infection, that they are inversely correlated to phenotypic markers of immune activation, and that they have a functional role for inhibiting bystander immune activation, while having a minimal impact on proliferative activities of HIV-1-specific T cells. Overall, these data suggest that HLA-G expressing Treg cells have important and unique functions for balancing and fine-tuning anti-viral immune activity and bystander immune activation during HIV-1 infection.

HLA-G positive Treg cells represent a relatively recently discovered group of suppressive T cells that can inhibit the activation and proliferation of T cells after TCR triggering with CD3/CD28 antibodies. But how HLA-G<sup>+</sup> Treg cells functionally compare to classical Treg subsets in terms of their ability to suppress virus-specific T cells or TCR-independent bystander activation of lymphocytes remained unclear. Our data show that HLA-G<sup>+</sup> Treg cells do not inhibit proliferation of HIV-1 and CMV-

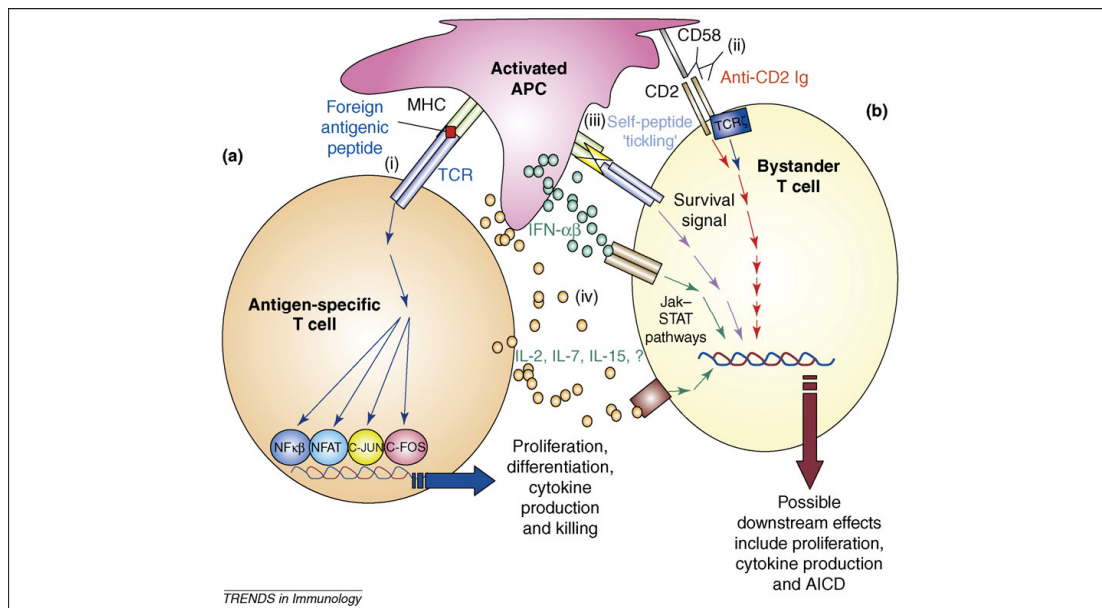
specific T cells, in comparison to classical Treg cells, which can suppress T cell proliferation by 30-40%. In contrast, we observed an augmented ability of these Treg cells to inhibit TCR-independent bystander activation of T cells, using an assay that excludes TCR cross-reactivity as a possible source of activation in heterologous T cells. Together, these results suggest that HLA-G<sup>+</sup> Treg cells differ from alternative Treg populations by a unique profile of suppressive functions that may allow for reducing bystander immune activation, while simultaneously minimizing inhibitory effects on virus-specific T cell immune responses. These cells might therefore be of particular benefit during progressive HIV-1 infection.

This work demonstrates that in contrast to classical Treg cells, HLA-G expressing Treg population progressively decline during advanced HIV-1 infection. This selective loss of HLA-G<sup>+</sup> CD4 Treg cells during advanced HIV-1 infection might represent an important contributory factor to the immune overactivation during progressive HIV-1 infection. The selective depletion of HLA-G Treg cells during progressive HIV-1 infection may be related to their increased susceptibility to HIV-1 infection, which is likely due to enhanced expression of the viral co-receptors CCR5 and CXCR4 demonstrated in this study. An upregulation of these chemokine receptors may also lead to elevated sequestration of HLA-G<sup>+</sup> Treg cells into inflamed tissues, where these cells were indeed preferentially observed in previous investigations (1). However, in our study, we did not find any positive evidence for a selective enrichment of HLA-G<sup>+</sup> Treg cells in lymphoid tissues, neither in HAART-treated nor in untreated HIV-1 patients. This observation in a limited number of patients does not exclude the possibility of tissue compartmentalization of HLA-G Treg cells in HIV-1 infection, and further studies are warranted to clarify

mechanisms responsible for the selective loss of HLA-G<sup>+</sup> CD4 Treg subset during progressive HIV-1 infection. In addition, the specific reason for the loss of HLA-G<sup>+</sup> CD8 Treg cells in untreated progressive HIV-1 infection remains unclear and warrants further investigation.

Over the recent years, HIV-1 infection has increasingly been recognized as a chronic inflammatory condition characterized by elevated T cell immune activation (42). The mechanisms leading to this abnormal immune activation are most likely multifactorial and include direct stimulation of T cells by HIV antigens, as well as direct TCR-mediated activation of T cells by alternative viral and bacterial antigens that challenge the host during conditions of HIV-1 associated immune deficiency (43-50). These TCR-dependent signals lead to the activation of transcription factors such as NF- $\kappa$ B, NFAT, C-JUN and C-FOS, which in turn results in phenotypic changes and effector functions, such as proliferation, cytotoxicity, cytokine production, and apoptosis (Figure 2-30).

Alternatively, T cells can be activated by bystander activation, defined as “the activation of a T cell to produce phenotypic or functional changes through a mechanism independent of specific TCR stimulation” (26). It is often triggered by other cell-surface molecule (such as CD2) ligation or certain cytokines. The most well known bystander-related cytokines include IL-2, IL7, IL-12, IL15, IL-18 and IFN- $\alpha/\beta$  (24, 51-53) (Figure 30). These cytokines, produced by antigen-presenting cells, T cells, or stromal cells, induce activation and proliferation signals in the target cells through less well-characterized pathways.



**Figure 2-30.** Mechanisms for direct (a) and bystander (b) T-cell activation. *Source: Bangs SC, et al: Bystander T cell activation – implications for HIV infection and other diseases. Trends Immunol (2006).*

Although TCR-independent bystander immune activation does not seem to play a significant role under physiologic conditions, increasing data suggest that bystander activation represents a major driving factor for pathological immune activation during progressive HIV-1 infection. For instance, bystander-activation-related cytokines, interferon- $\alpha/\beta$ , IL-2 and IL-15, are all increased in HIV-1 infection and represent independent and accurate predictors of disease progression (54). Moreover, the majority of activated T cells in HIV-1 infected patients typically do not exhibit phenotypic markers of recent TCR stimulation (55), suggesting that their activation occurred by TCR-independent processes. In addition, activation of T cells specific for Influenza virus has been documented during HIV-1 infection in the absence of serological evidence of Influenza co-infection, or detectable TCR cross-reactivity between HIV-1 and Influenza antigens (48).

Interestingly, our data suggest that T cells activated by bystander

mechanisms have a higher susceptibility to inhibitory effects of HLA-G<sup>+</sup> Treg, likely because they do not downregulate the HLA-G receptor LILRB1 in a similar way as T cells activated by TCR triggering. The exact reason why CD25<sup>hi</sup> Treg cells do not reduce bystander activation as much as they do for the directed-stimulated activation is unclear. One of the mechanisms that CD25<sup>hi</sup> Treg (but not HLA-G Treg) uses to suppress target cells is to induce TGF- $\beta$  secretion (56, 57). Interestingly, it was reported that TGF- $\beta$  receptor expression increased upon immune activation (58), as a part of the negative control loop. Noticeably, the directly-stimulated T cell population in our experiment expressed a significantly higher level of activation markers than that of the bystander cells (data not shown), indicating that they might possess a higher expression of surface TGF- $\beta$  receptor, thus were more susceptible to CD25<sup>hi</sup> Treg's suppressive effect. However whether or not a differential expression of TGF- $\beta$  receptor exists, and how much does it contribute to the different levels of regulation by CD25<sup>hi</sup> Treg on bystander vs. directly-stimulated cells warrants further investigation. Taken together, these observations indicate that TCR-dependent and TCR-independent mechanisms of immune activation are associated with altered susceptibilities to classical and non-classical Treg populations, and shed new light on target cell characteristics that influence inhibitory effects of Treg cells. By selectively inhibiting the deleterious effects of TCR-independent bystander activation, the HLA-G<sup>+</sup> Treg subset may provide a previously unrecognized form of immune protection against HIV-1 associated disease manifestations.

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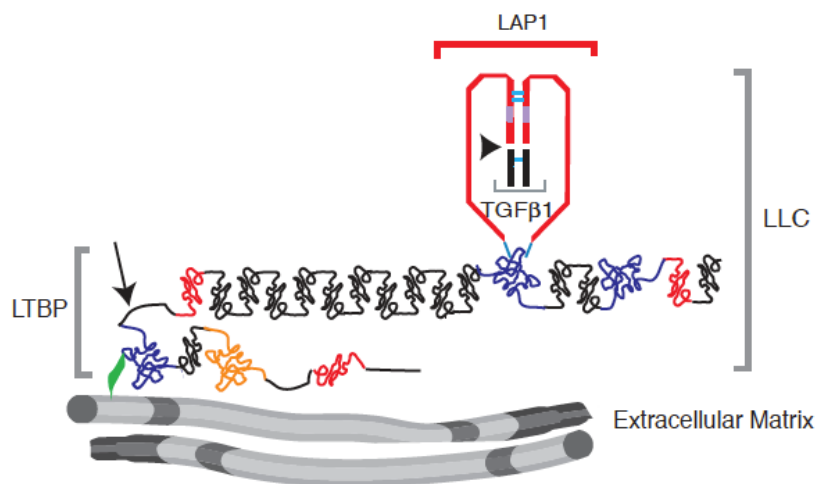
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## **Chapter 3**

# **Comprehensive characterization of LAP- expressing regulatory T cells in HIV-1 infection**

## Introduction

One member of the emerging non-classical regulatory T cell populations is characterized by surface expression of latency-associated peptide (LAP) (1). LAP is the amino-terminal domain of TGF- $\beta$ 1 precursor. After being cleaved from the precursor, it remains non-covalently connected with TGF- $\beta$  to prevent uncontrolled activation of the cognate TGF receptors (2, 3).



**Figure 3-1.** LAP covalently binds to latent form of TGF- $\beta$ . Source: Annes, J et al, *Making sense of latent TGFbeta activation. J Cell Sci, 2003*

Unlike HLA-G-expressing Treg cells, which display minimal expression of CD25, LAP was first found on a subset of classical CD25<sup>hi</sup> CD4Treg cells. This LAP<sup>+</sup> Treg subset expressed elevated levels of CTLA4, TGF- $\beta$  and TGF- $\beta$  receptor, and appeared to be more suppressive than their LAP-negative CD25<sup>hi</sup> counter partners (4). Later it was found that LAP itself was sufficient to define a novel CD4 Treg population; these LAP<sup>+</sup> CD4 T cells lacked FoxP3 expression but were able to inhibit proliferative activities of T lymphocytes *in vitro* and *in vivo* (1). In addition, LAP<sup>+</sup> Treg cells were different from the classical Treg populations in the surface expression level of TGF- $\beta$ RII and CD69, as well as cytokine secretion profile upon activation (1). The numeric

distribution and functional role of LAP<sup>+</sup> CD4 Treg cells during HIV-1 infection is not known.

In the present study, we systematically analyzed the expression and function of LAP-expressing Treg cells in patients with different stages of HIV-1 infection. Our results indicated a reduction of LAP<sup>+</sup> CD4 Treg cell numbers, but not proportions, in individuals with progressive HIV-1 disease. Moreover, similar to classical CD25<sup>hi</sup>CD127<sup>lo</sup>CD4 Treg cells, LAP<sup>+</sup> CD4 Treg subset was able to suppress HIV-1-specific proliferation and TCR-driven immune activation, but not TCR-independent bystander activation.

## **Materials and Methods**

### *Study participants*

HIV-infected patients and HIV-1 seronegative control persons were recruited according to protocols approved by the Institutional Review Board of the Massachusetts General Hospital in Boston.

We have included in the study 5 patient groups with various status of HIV infection: 10 patients with primary infection and seroconversion within 3 months prior to recruitment (acutely-infected patients), 15 treatment-naïve HIV-1 infected individuals with chronic progressive infection (progressors), 9 HIV-1 infected persons successfully treated with HAART, 17 patients with spontaneous control of HIV-1 replication (controllers), and 11 non-HIV-infected healthy individuals (HIV negative).

Among acutely-infected patients, the viremia ranges from 36,600-982,000 copies/ $\mu$ l, with a median of 152,400 copies/ml; CD4 T cell count varies from 265 to 1047/ $\mu$ l with a median of 489/ $\mu$ l. For chronic progressors, the viral load varies from 27,700-112,000 copies/ml, with a median of 50,600 copies/ml; CD4 T cell count varies from 229-652/ $\mu$ l, with a median of 456/ $\mu$ l. As for HAART-treated patients, all of them have viremia <50 copies/ml, and their CD4 T cell count ranges from 415-1493/ $\mu$ l with a median of 803/ $\mu$ l. For controllers, all of them have viral load <1,000 copies/ml, and CD4 T cell count varies from 440-1415/ $\mu$ l, with a median of 839/ $\mu$ l.

### *Immunophenotypic analysis*

Peripheral blood mononuclear cell (PBMC) were isolated from whole blood using Ficoll density centrifugation. PBMC were stained with LIVE/DEAD cell viability dye (Invitrogen) and monoclonal antibodies directed against CD4, CD25, CD127,



CD45RA, CCR7 (BD Biosciences), CD57 and PD-1 (Biolegend), CD8 (Invitrogen) and LAP (Clone 27232, R&D systems). After incubation for 20 minutes at room temperature, cells were fixed with PBS containing 0.5% fetal calf serum and 1% formaldehyde. Cells were acquired on LSR Fortessa™ Cell Analyzer (BD Biosciences) using DiVA software. Data were analyzed using FlowJo software (Tree Star).

#### *Cell isolation and sorting*

CD4 or CD8 T cells were isolated using a negative cell purification kit (Stemcell), according to the manufacturer's instructions. Cell purity was >90% in all cases. Subsequently, Fluorescence activated cell sorting was used to divide bulk CD4 T cells into four subgroups: LAP<sup>-</sup> CD25<sup>hi</sup> CD4 T cells, LAP<sup>+</sup> CD25<sup>lo</sup> CD4 T cells, and LAP<sup>-</sup> CD25<sup>lo</sup> CD4 T cells. For this purpose, CD4 cells were labeled with the respective monoclonal antibodies and viability dye, washed, and sorted on a FACSaria instrument (BD Biosciences) at 70 pounds per square inch.

#### *Proliferation Assay*

PBMC from HIV-1 infected individuals were stained with 0.25  $\mu$ M carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) and mixed with sorted autologous Treg populations or control T cells without regulatory activity at ratios of 4:1. Afterwards, cells were stimulated with a pool of overlapping peptides spanning the clade B consensus sequences of HIV-1 Gag (2  $\mu$ g/ml per peptide). After incubation for 6 days in RPMI 1640 medium supplemented with 2 mM l-glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 10 mM Hepes, and 10% heat-inactivated FCS, cells were washed, stained with viability dye, CD4 and CD8

antibodies, fixed and acquired on a LSR Fortessa flow cytometer. Suppression of T cell proliferation by Tregs was calculated as: (T cell proliferation (%) in the non-Treg co-culture – T cell proliferation (%) in the Treg co-culture)/T cell proliferation (%) in the non-Treg co-culture.

#### *Intracellular Cytokine Staining*

Purified CD3 T responder cells from HIV-1 patients were stained with CFSE as described above and subsequently mixed with sorted autologous Treg populations or control CD4 T cells at a ratio of 2:1. Cells were then stimulated with a pool of overlapping peptides spanning HIV-1 Gag (2µg/ml per peptide) in the presence of antibodies directed against CD28 and CD49d (2µg/ml). Cells were incubated for 6h at 37°C, and brefeldin A was added at 5µg/ml after the first hour of incubation. Afterwards, cells were stained with viability dye and antibodies against CD4 and CD8, fixed and permeabilized using a commercial kit (Caltag, Burlingame, CA), and subjected to intracellular cytokine staining with monoclonal antibodies against interferon-γ (Becton Dickinson). Following final washes, cells were acquired on a LSR Fortessa instrument. The proportion of IFN-γ<sup>+</sup> or IL-2<sup>+</sup> CD4 and CD8 T cells was determined after gating on CD3<sup>+</sup> CFSE<sup>+</sup> responder T cells, using FlowJo software.

#### *Assessment of bystander activation*

Purified CD3<sup>+</sup> responder T cells from healthy individuals were mixed with sorted autologous Tregs populations or autologous control CD4 T cells without regulatory activities at a ratio of 2:1. Following stimulation of cells with SEB, cells were incubated at 37°C for 4 days. Afterwards, cells were stained with antibodies

against CD4, CD8, CD38, HLA-DR and Vβ13.1 and viability dye before being subjected to flow cytometric acquisition on a LSR Fortessa instrument. The surface expression of activation markers in responder T cells was analyzed after gating on CD3<sup>+</sup> events. Treg-dependent suppression of bystander activation was calculated as: (CD38/HLA-DR -expressing T cells (%) in the non-Treg co-culture – CD38/HLA-DR -expressing T cells in the Treg co-culture)/CD38/HLA-DR -expressing T cells (%) in the non-Treg co-culture).

#### *Statistical analysis*

Data are expressed as mean and standard deviation, or as box and whisker plots indicating the median, the 25% and 75% percentile and the minimum and maximum of all data. Differences between different cohorts or different experimental conditions were tested for statistical significance using analysis of variance (ANOVA), Mann-Whitney U test, Wilcoxon signed rank test, or paired/unpaired t tests, as appropriate. Spearman correlation was used to assess the association between two variables. A p-value of 0.05 was considered significant.

## Results and Discussion

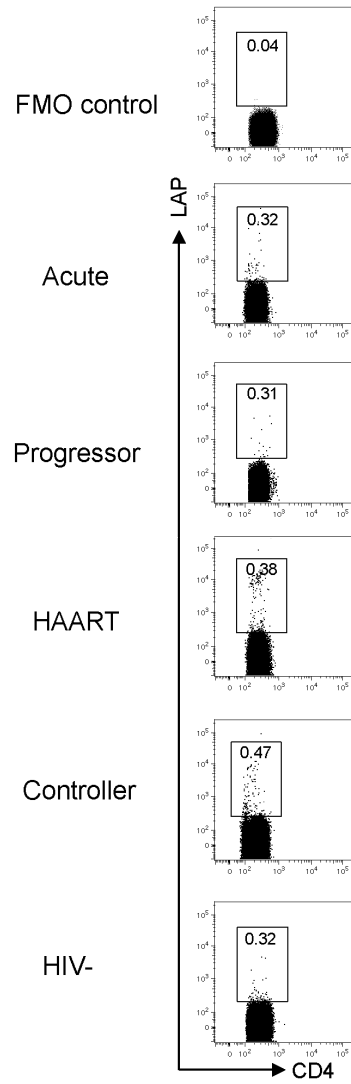
### *Decreased numbers of LAP<sup>+</sup> CD4 T cells in HIV-1 infection*

CD4 T cells expressing LAP, a membrane-bound form of TGF- $\beta$ , have recently been characterized as an alternative, Foxp3-negative population of lymphocytes with immunosuppressive properties (1). To determine whether this non-classical population of regulatory cells is involved in HIV-1 disease pathogenesis, we analyzed the frequency of LAP<sup>+</sup> CD4 T cells in our study cohorts.

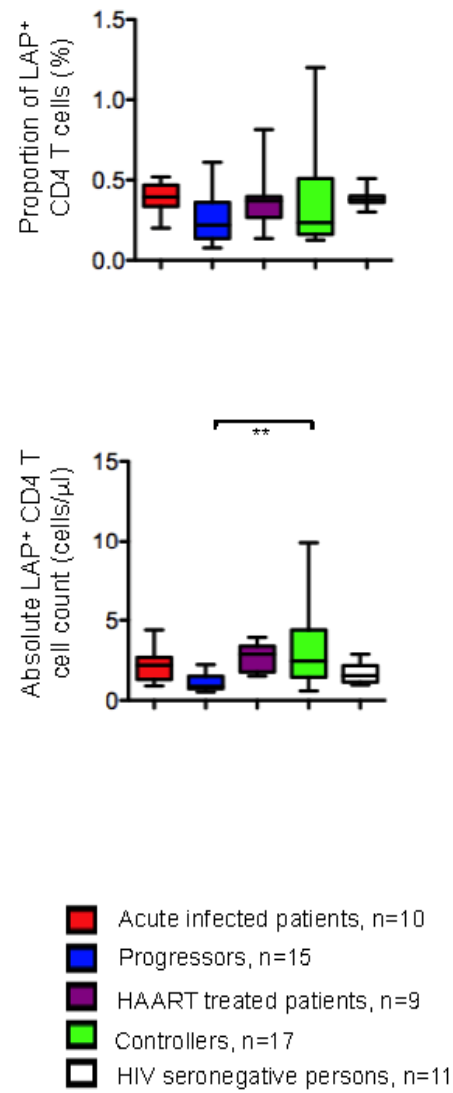
We observed no differences in proportions of LAP-expressing CD4 T cells across all HIV-1 infected cohorts (Figure 3-2, 3-3). Proportions of LAP<sup>+</sup> CD8 T cells, which may also have regulatory activities (1), did not differ between our study groups (Figure 3-4, 3-5). LAP<sup>+</sup> T cell proportions were not significantly correlated with CD4 T cell count (Figure 3-6), or plasma viral load (Figure 3-7). LAP-expressing CD4 T cell frequencies and absolute counts were positively associated with immune activation level, which indicated that it might serve as an activation marker in HIV-1 infection (Figure 3-8).

We next examined the differentiation and maturation status of LAP<sup>+</sup> T cells. We did not find a substantial difference between LAP<sup>+</sup> T and bulk T cells in terms of T cell subset distribution, although LAP<sup>+</sup> CD8 T cells appeared to be slightly overrepresented in central-memory cells during HIV-1 infection (Figure 3-9, 3-10). No differences were found between the surface expression of PD-1 and CD57 on LAP<sup>+</sup> T cells and bulk T cells (Figure 3-11, 3-12).

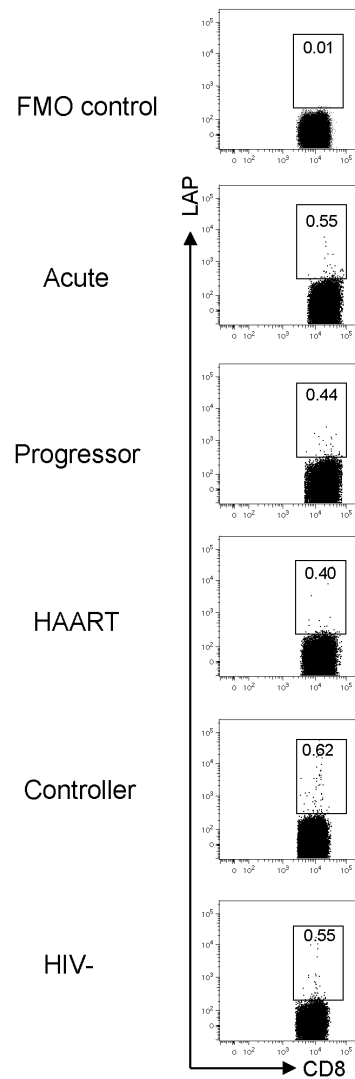
Taken together, these results suggested a lack of direct correlation of LAP-expressing T cells and HIV-1 disease outcome. However it did not exclude the possibility that these cells could still influence specific functional aspects of the immune system during the infection course.



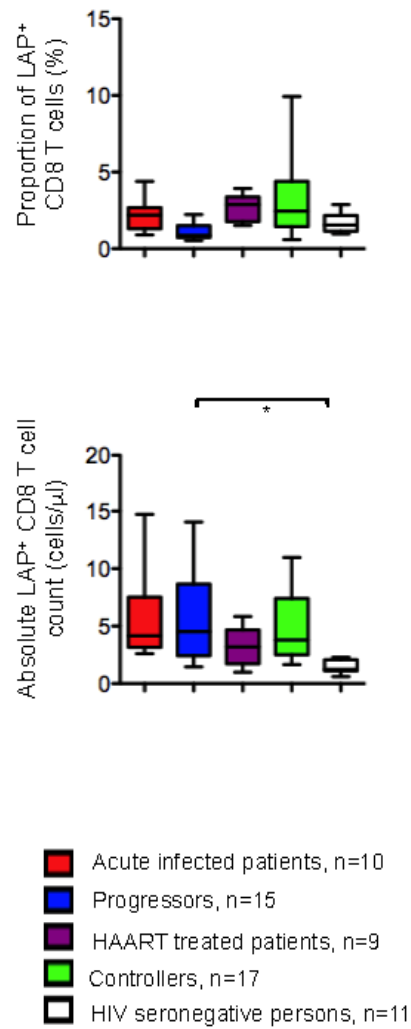
**Figure 3-2.** Representative flow plots reflect proportions of LAP<sup>+</sup> CD4 T cells in indicated study populations.



**Figure 3-3.** Box and Whisker plots summarize the relative proportions and absolute counts of LAP<sup>+</sup> CD4 T cells in indicated study populations. \*\*: p<0.01.

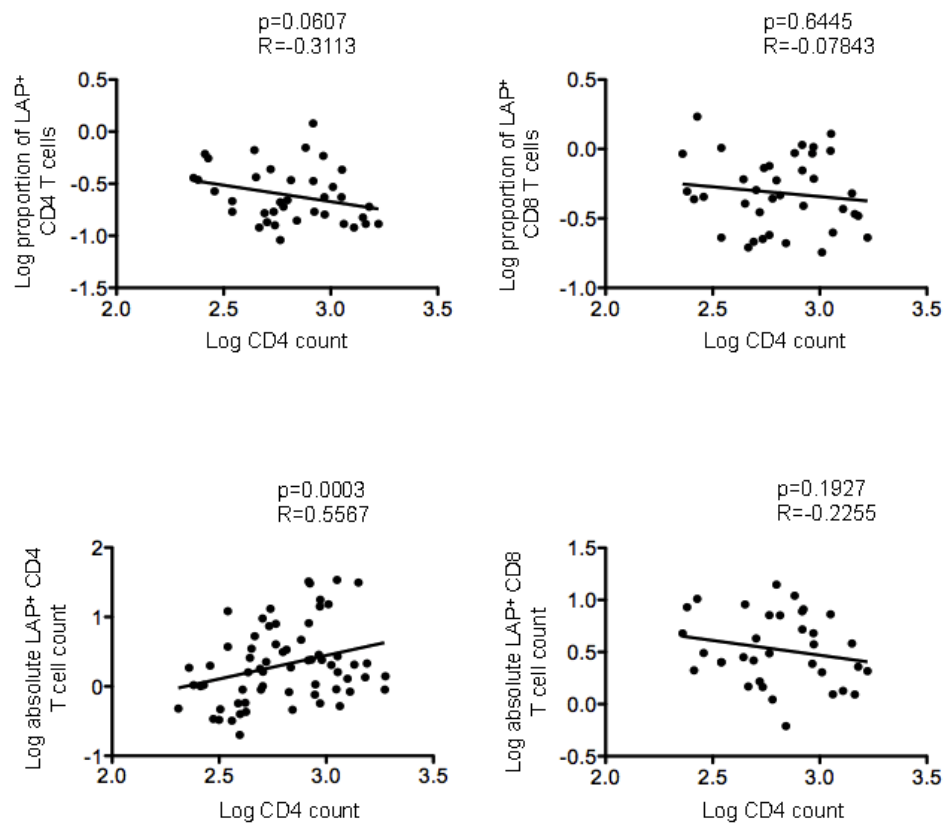


**Figure 3-4.** Representative flow plots reflect proportions of LAP<sup>+</sup> CD8<sup>+</sup> T cells in indicated study populations.

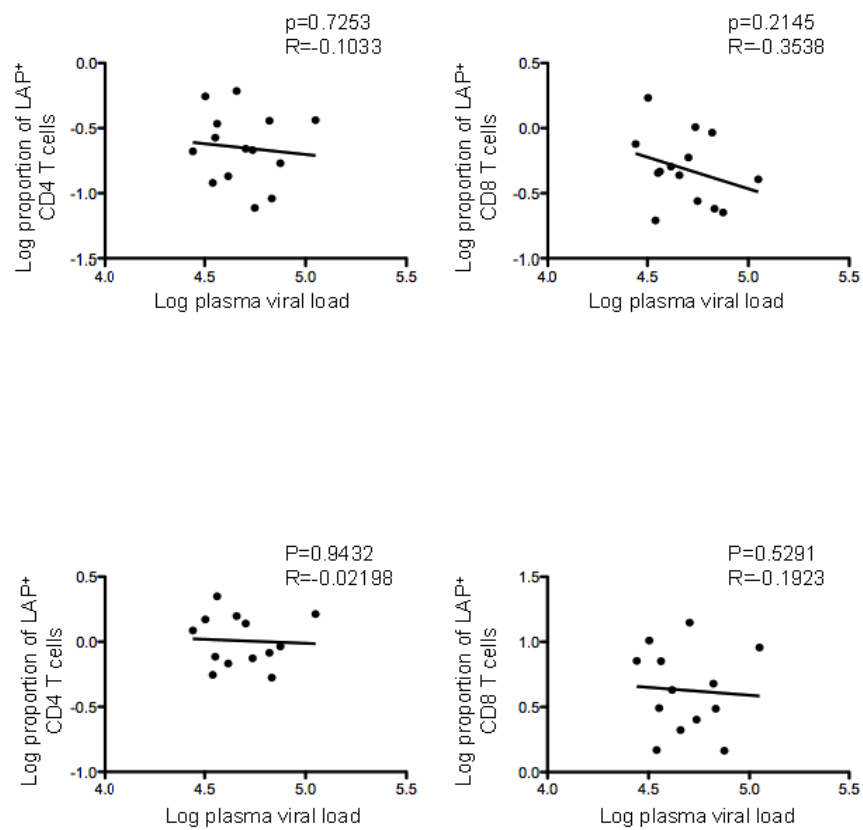


**Figure 3-5.** Box and Whisker plots summarize the relative proportions and absolute counts of LAP<sup>+</sup> CD8 T cells in indicated study populations. \*:p<0.05.

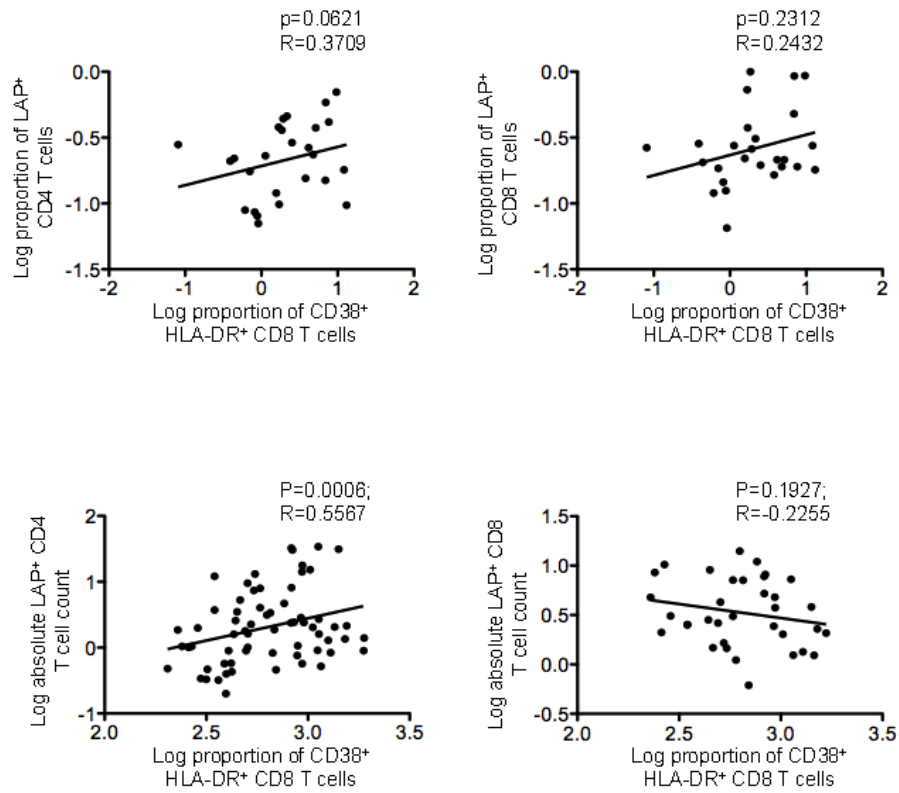




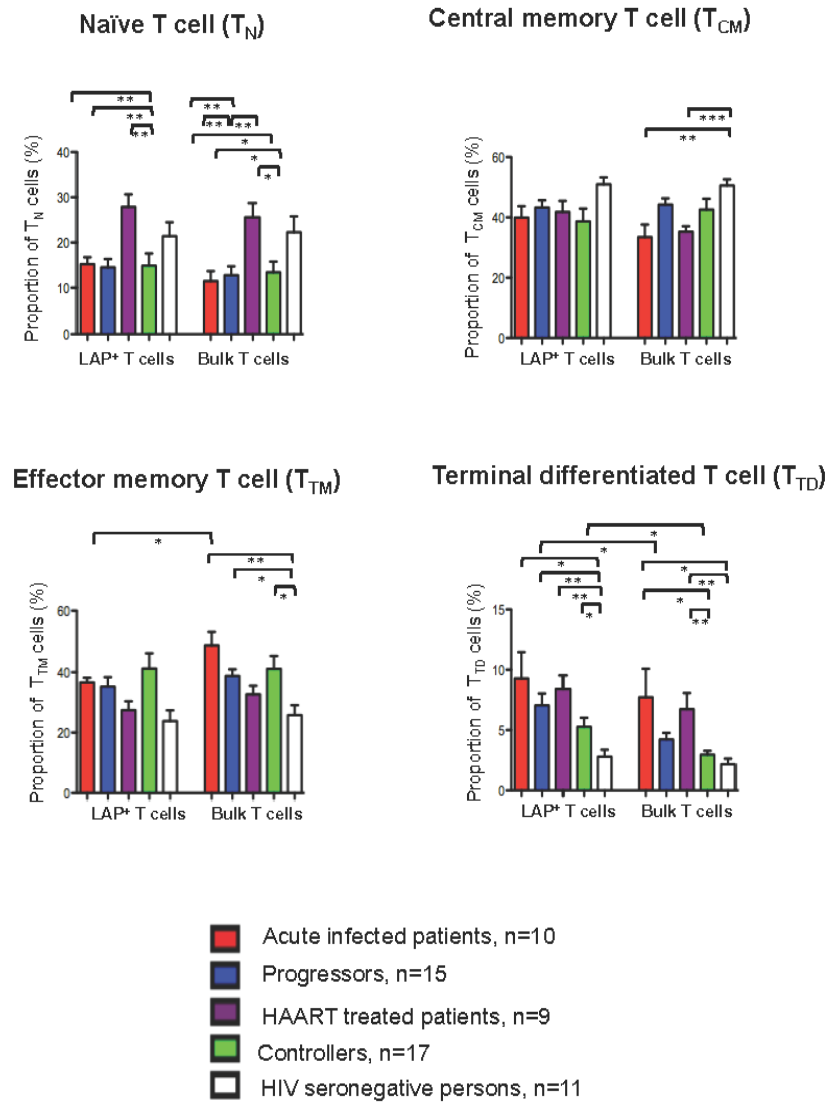
**Figure 3-6.** Correlations between proportions of LAP<sup>+</sup> T cells and CD4 T cells counts in controllers (n=16), progressors (n=14) and HIV- persons (n=7).



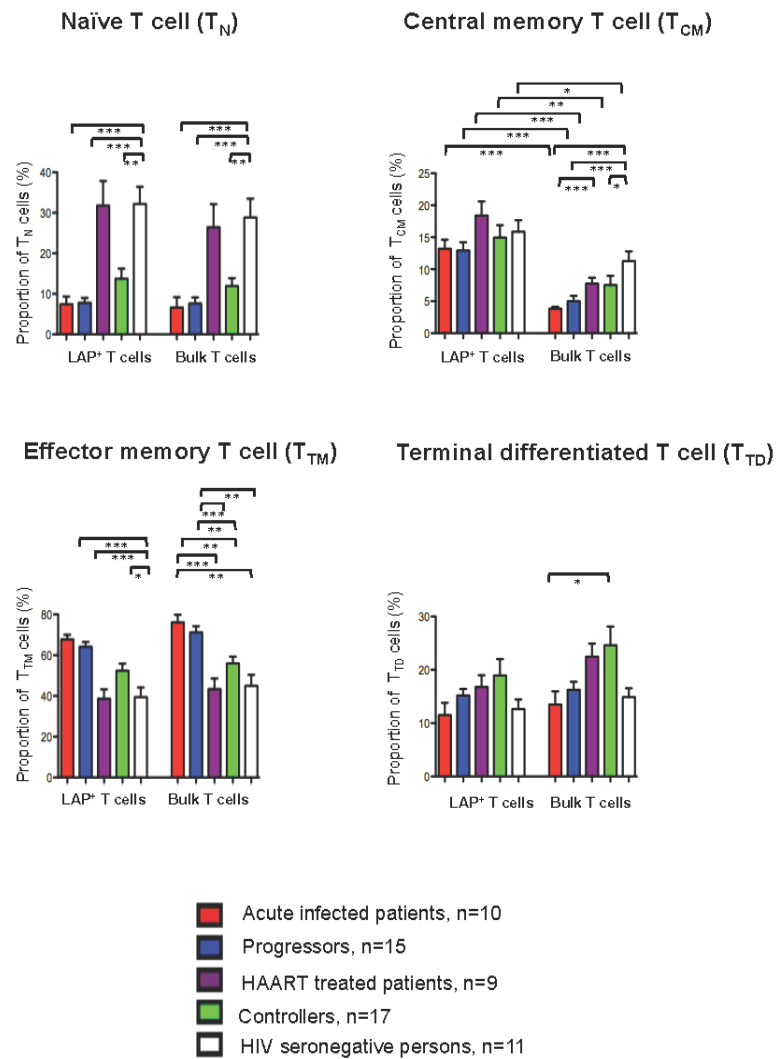
**Figure 3-7.** Correlations between proportions of LAP<sup>+</sup> T cells and plasma viral load in progressors (n=14).



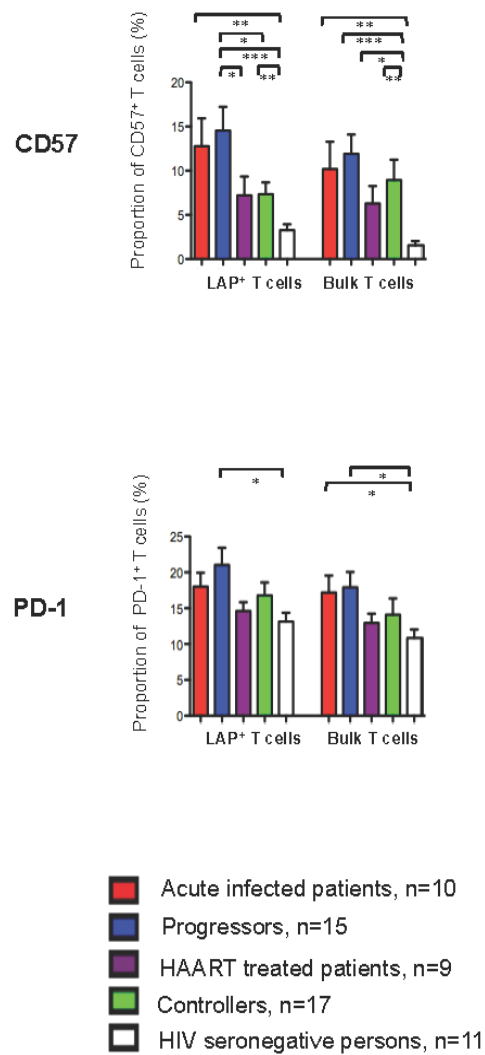
**Figure 3-8.** Correlations between proportions of LAP<sup>+</sup> T cells and T cell immune activation level, determined by surface expression of CD38 and HLA-DR in controllers (n=13), progressors (n=7), and HIV- persons(n=6).



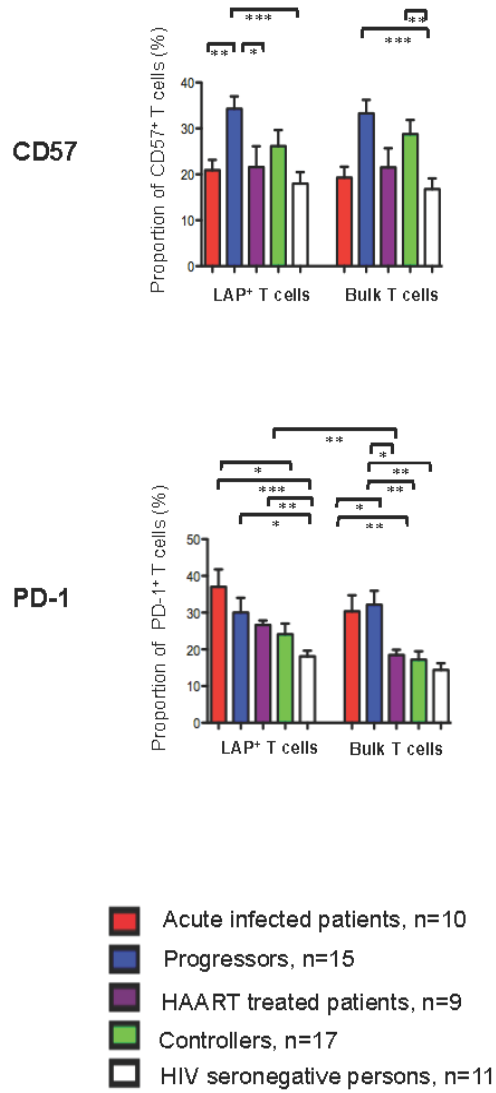
**Figure 3-9.** T cell subset distribution of LAP-expressing CD4 T cells in indicated study populations. Data from corresponding bulk T cell populations are also shown for reference purposes. \*:p<0.05; \*\*:p<0.01; \*\*\*:p<0.001.



**Figure 3-10.** T cell subsets distribution of LAP-expressing CD8 T cells in indicated study populations. Data from corresponding bulk T cell populations are also shown for reference purposes. \*:p<0.05; \*\*:p<0.01; \*\*\*:p<0.001.



**Figure 3-11.** Surface expression of CD57 and PD-1 in LAP<sup>+</sup> expressing CD4 T cells in indicated study cohorts. Data from corresponding bulk T cell populations are also shown for reference purposes. \*:p<0.05; \*\*:p<0.01; \*\*\*:p<0.001.



**Figure 3-12.** Surface expression of CD57 and PD-1 in LAP<sup>+</sup> expressing CD8 T cells in indicated study cohorts. Data from corresponding bulk T cell populations are also shown for reference purposes. \*:p<0.05; \*\*:p<0.01; \*\*\*:p<0.001.

*LAP-expressing Treg weakly inhibit functional properties of HIV-1-specific CD8 T cells*

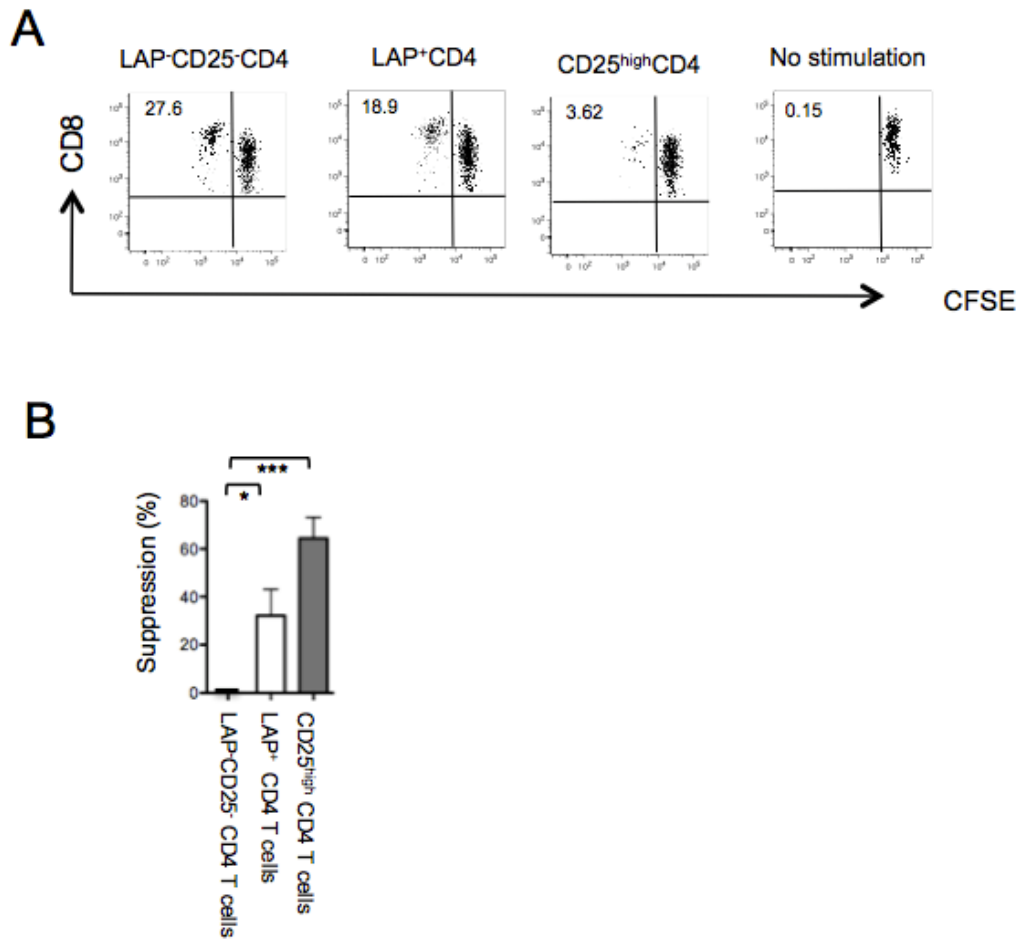
It has been shown that LAP-expressing CD4 T cells suppress poly-clonal T cell proliferation (1), but their functional effects on HIV-1-specific T cells remain unclear. To investigate this, CFSE-labeled PBMCs from HIV-1 patients were stimulated with a pool of overlapping HIV-1 gag peptides. The stimulated cells were then individually mixed with sorted autologous LAP<sup>+</sup> CD25<sup>-</sup> CD4 T cells, LAP<sup>-</sup> CD25<sup>+</sup> CD4 T cells, or a control population of LAP<sup>-</sup> CD25<sup>-</sup> CD4 conversional T cells. Subsequently, proliferation of HIV-specific CD8 T cells was monitored after six days of culture.

The results confirmed the strong suppressive effects of classical CD25<sup>+</sup> Treg cells on the proliferative activities of HIV-1-specific cells (Figure 3-13) (5). Interestingly, LAP-expressing Treg subset also seemed to be able to reduce the proliferation of HIV-1-specific CD8 T cells, although this effect was attenuated as compared to that of CD25<sup>hi</sup>CD4 Treg cells (Figure 3-13).

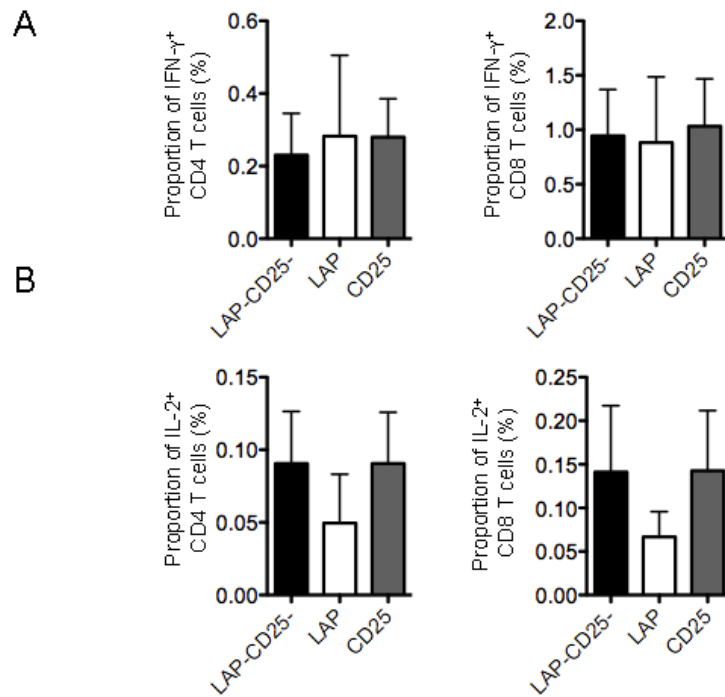
To assess whether LAP<sup>+</sup> CD4 T cells influence the cytokine secretion properties of HIV-1-specific T cells, isolated CD3 T cells from HIV-1-infected persons were stimulated with overlapping gag peptides in the presence of the LAP<sup>+</sup> CD25<sup>-</sup> CD4 T cells, LAP<sup>-</sup> CD25<sup>+</sup> CD4 T cells, or LAP<sup>-</sup> CD25<sup>-</sup> control cells; after 16 hours of incubation, HIV-specific secretion of interferon- $\gamma$  and IL-2 was monitored by flow cytometry. As summarized in Figure 3-14, cytokine secretion of HIV-1-specific CD8 T cells did not differ following co-incubation with any of the Treg populations or negative control cells.

Taken together, these data show that LAP-expressing Tregs have limited effects on the functional activities of HIV-1-specific CD8 T cells.





**Figure 3-13.** LAP-expressing Treg cells weakly inhibit proliferative activities of HIV-1 stimulated cytotoxic T cells. (A): Representative dot plots reflect proliferative activities of HIV-1 CD8 T cells following incubation with indicated autologous Treg subsets or LAP<sup>-</sup> CD25<sup>-</sup> control cells. (B): Cumulative data from n=8 study subjects indicate Treg mediated suppression of CD8 T cell proliferating. \*:p<0.05; \*\*\*:p<0.001.



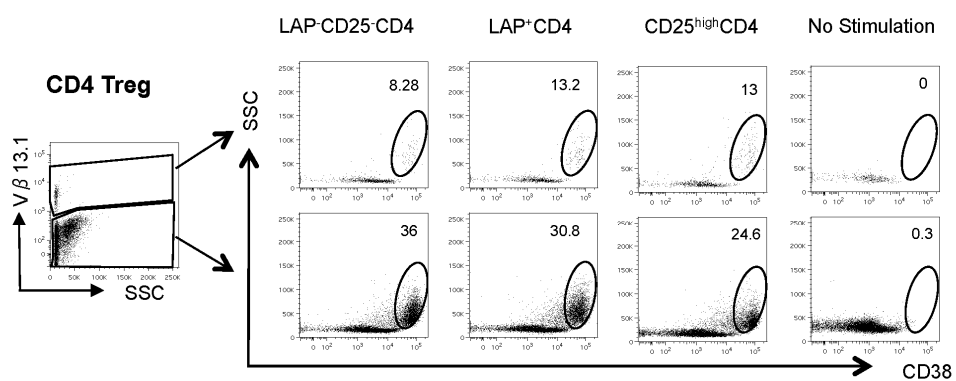
**Figure 3-14.** LAP-expressing Treg cells do not affect cytokine secretion properties of HIV-1- specific T cells. (A): Cumulative data from n=5 study subjects indicate the proportion of IFN<sup>+</sup> CD4 and CD8 T cells after co-incubation with indicated Treg subsets or control CD4 T cells. (B): Cumulative data from n=5 study subjects indicating the proportion of IL-2<sup>+</sup> CD4 and CD8 T cells after co-incubation with indicated Treg subsets or control CD4 T cells.

*LAP expressing Treg cells do not inhibit bystander activation*

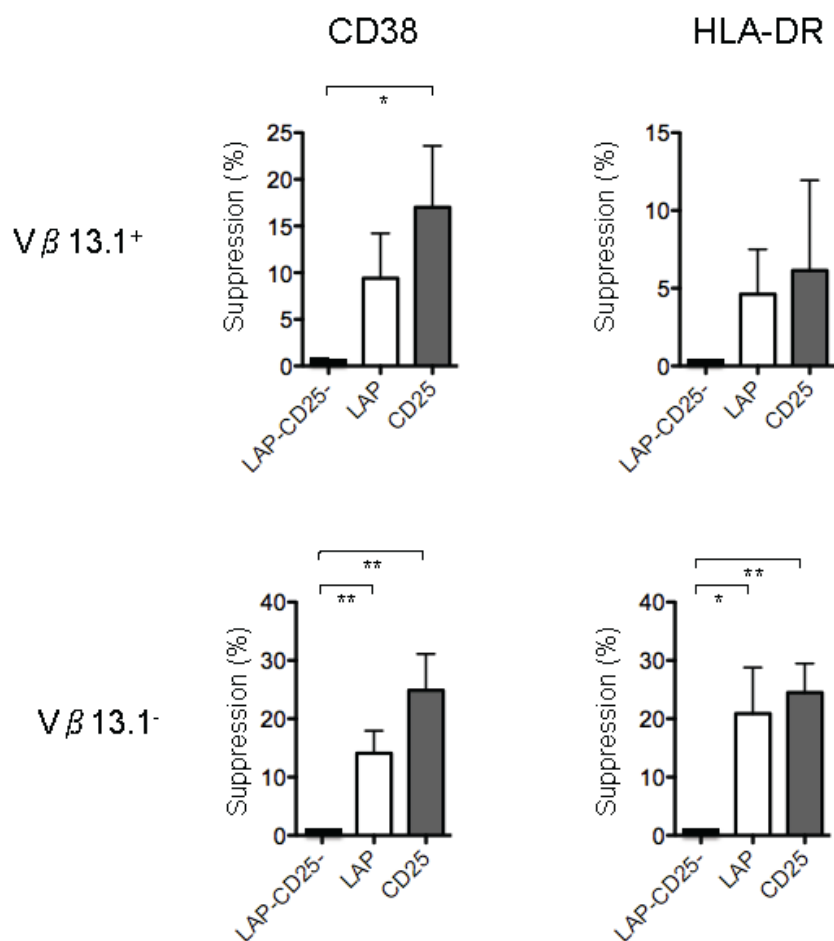
To further explore the role of LAP-expressing Treg cells in HIV-1 disease pathogenesis, we focused on how they influence T cell activation. We employed a similar experimental system as in the HLA-G study: we stimulated responder T cells with SEB, and co-cultured them with autologous populations of sorted LAP<sup>+</sup> CD25<sup>-</sup> CD4<sup>+</sup> Treg cells, LAP<sup>-</sup> CD25<sup>+</sup> CD4<sup>+</sup> Treg cells, or a control LAP<sup>-</sup> CD25<sup>-</sup> CD4<sup>+</sup> population. On day 4 of culture, measured surface expression of CD38 and HLA-DR on Vβ13.1<sup>+</sup> and Vβ13.1<sup>-</sup> T cells.

Interestingly, we did not observe the same effect as we did with HLA-G-expressing Treg cells. Rather, LAP-expressing CD4<sup>+</sup> cells acted more like CD25<sup>hi</sup> classical Treg cells in that they selectively suppressed the TCR-dependent activation of the Vβ13.1<sup>-</sup> population, though to a lesser extent than classical Treg subset (Figure 3-15, 3-16).

Overall these data suggested that LAP-expressing CD4<sup>+</sup> Treg cells have limited functions for regulating HIV-1 disease progression. These cells did not affect HIV-1-specific cytokine secretion or bystander activation, while moderately suppress HIV-1 specific CD8 T cell proliferation. These functional data are in line with these cells' weak correlation to HIV-1 disease outcome.



**Figure 3-15.** Representative flow cytometry dot plots reflect CD38 expression on Vβ13.1<sup>+</sup> and Vβ13.1<sup>-</sup> responder T cells. Responder cells were isolated from HIV seronegative individuals and activated by SEB, in the presence of autologous CD4 Treg or conventional CD4 T cells.



**Figure 3-16.** Cumulative data represent relative suppression of CD38 and HLA-DR on  $V\beta 13.1^+$  and  $V\beta 13.1^-$  responder T cells following exposure to SEB and co-incubation with CD4 Treg cell populations or control T cells. Data from n=8 study subjects are shown. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

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## **Chapter 4**

### **Conclusions**

Studying the role of regulatory T cells during HIV-1 infection may be of critical importance, given the demonstrated association of immune activation with HIV disease progression, and that these cells may have a critical influence on immune defense and immune activation in HIV-1 patients. On the one hand, one could hypothesize that Treg cells could suppress host immune responses elicited against the virus, therefore accelerating disease progression; on the other hand, Treg cells may lead to a better disease outcome by actively inhibiting over-activation of the immune system, which may cause more damage to the organisms than the infection itself. Classical Treg cells appear to be expanded during chronic HIV-1 infection, and contribute to acceleration of HIV-1 infection by inhibiting HIV-1-specific CTL responses (1-6).

This thesis aims to determine the overall role of non-classical (Foxp3 negative), HLA-G-expressing (Chapter 2) and LAP-expressing (Chapter 3) Treg cells in HIV-1 infection. To this end, we compared the relative proportions and absolute numbers of HLA-G- and LAP-expressing Treg cells across patients with different disease stages. We also correlated the frequencies of these Treg cells with prognostic markers, such as CD4 T cell counts and immune activation levels. Our results suggest a reduction in both of the frequency and number of HLA-G<sup>+</sup> Treg cells in progressive HIV-1 disease courses, which is strongly correlated with low CD4 T cell counts and high levels of immune activation. In contrast, no apparent correlation was found between frequencies of LAP<sup>+</sup> Treg cells and HIV-1 disease outcomes. Either way, these associations clearly distinguish both non-classical Treg populations from the classical Treg cells.

Furthermore, our functional studies indicate that HLA-G<sup>+</sup> Treg cells specifically inhibit TCR-independent bystander activation, while having minimal impact on TCR-driven T cell activation. Because bystander activation is prominent in chronic HIV-1 infection and can directly lead to cell injury and cell death (7-9), the ability to inhibit



such processes could have a significant contribution to HIV-1 disease control. More importantly, this suppression of bystander activation is not achieved at the cost of inhibiting HIV-1-specific immune responses. In fact, HLA-G<sup>+</sup> Treg cells exhibit a minimal effect, if any, on HIV-1-specific helper T cell and cytotoxic T cell response compared to CD25<sup>hi</sup> Treg cells'. This distinctive ability to fine-tune anti-viral immunity may explain the strong positive association between the frequencies of HLA-G-expressing cells and preservation of sufficient CD4 T cell counts in untreated HIV-1 patients.

We have shown that HLA-G-expressing Treg cells are able to suppress bystander immune activation of conventional T cells. Moreover, this suppression is associated with the persistent expression of LILRB1 (HLA-G's receptor) on these bystander cells. In the mean time, TCR-dependent T cell activation leads to LILRB1 downregulation, and makes cells resistant to immunomodulatory effects of HLA-G<sup>+</sup> Treg cells. Further studies involving siRNA knock-down or protein blocking of LILRB1 could confirm its role for mediating suppressive effects of HLA-G Treg cells. In addition, it may be useful to explore downstream pathways that are involved in modulating cells activated by TCR-independent, "bystander" mechanisms.

Interestingly, we have shown that similar to conventional CD25<sup>hi</sup> T cells (10, 11), HLA-G-expressing CD4 Treg cells express high levels of the HIV-1 co-receptors CXCR4 and CCR5, and are highly susceptible to HIV-1 infection. This susceptibility may explain the reduction of HLA-G<sup>+</sup> CD4 Treg cells seen in chronic progressors, whose viral loads are significantly higher and more persistent than those of controllers. In addition, the specific reason for the loss of HLA-G<sup>+</sup> CD8 Treg cells in untreated progressive HIV-1 infection remains unclear and warrants further investigation.

Our investigations focused mostly on the functional quantitative changes of HLA-G-expressing Treg cells. It is possible that the quality of these cells also change during the course of HIV infection. For example, it was shown that in HIV-infected patients, whose Treg cell number was reduced, CD4 effector T cells became more sensitive to CD25<sup>hi</sup> CD4 Treg cells' immunoregulation (12). We could analyze if HLA-G Treg cells also have this "compensating" mechanisms, by isolating HLA-G Treg cells from HIV-1 infected patients and from healthy individuals, and comparing their suppressive efficiency.

The work presented in this thesis opens new questions that warrant future investigations. An important next step would be to see how these non-classical Treg cells behave *in vivo* in animal models of HIV-1 infection. Using this approach, one could address the following questions: Is depletion of HLA-G<sup>+</sup> Treg cells accelerating disease progression? Do their proportion/number correlate with disease outcome? Could they still suppress bystander activation while maintain a minimum impact on HIV-1-specific responses? As HIV-1 is a primate lentivirus that does not infect small laboratory animals like mice, most of the current knowledge about AIDS has come from studies of nonhuman primates, which support replication of SIV, the origin and a close relative of HIV-1 (13). Although the SIV/primate models are invaluable for studying the disease pathogenesis (14), the divergence between SIV and HIV-1, as well as the differences in the MHC class I alleles and TCR repertoire should nevertheless be noted. Because of these differences, the epitope-specific CTL responses directed against SIV in macaques may not be comparable to the CTL responses induced against human HIV-1. The recent generation of the humanized BLT (bone marrow, liver, thymus) mouse model may be useful for these investigations. The BLT model utilizes the

engraftment of human fetal thymic and liver tissues along with human fetal liver-derived CD34<sup>+</sup> hematopoietic stem cells (HSCs), which support the development of a relatively complete human immune system in a mouse. This system was shown to be capable of inducing very comparable HIV-1-specific T cell responses upon viral infection in humans (15). The result of this study may shed light on a previously unrecognized form of immune protection against HIV-1 associated disease manifestations *in vivo*.

Further, we have analyzed the regulatory properties of two non-classical Treg populations, HLA-G- and LAP- expressing Treg subsets, in HIV-1 disease progression. However, the role of other non-classical Treg cells, including but not limited to CD25<sup>+</sup>CD28<sup>-</sup> CD8<sup>+</sup>T cells (16),  $\gamma\delta$ -TCT<sup>+</sup> T cells (17), Tr1/Th3 T cells (18, 19), NKT cells (20), CD8<sup>+</sup> veto cells (21), and  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cells (22), remains to be determined. In addition, the roles of regulatory macrophages (23, 24) and tolerogenic dendritic cells (25, 26) in HIV-1 infection were not known. Investigations on these regulatory subsets may be helpful for understanding the complex immunoregulatory mechanisms associated with HIV-1 infection in humans.

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## Appendix

### List of Publications

**Li C**, Toth I, Schulze zur Wiesch J, Pereyra F, Rychert J, Rosenberg ES, van Lunzen J, Lichterfeld M, Yu XG. HLA-G<sup>+</sup> regulatory T cells reduce bystander immune activation in HIV-1 infection. *PLOS Pathogens*, in press.

Chen H\*, **Li C\***, Huang J\*, Cung T\*(\***co-first author**), Seiss K, Beamon J, Carrington M, Porter L, Burke P, Yang Y, Ryan B, Liu R, Weiss R, Pereyra F, Cress W, Brass A, Rosenberg E, Walker B, Yu X, Lichterfeld M. CD4 T cells from elite controllers resist HIV-1 infection by selective upregulation of p21 (cip-1/waf-1). *Journal of Clinical Investigation*, Vol.121(4): 1549–1560; April, 2011.

Vigneault F, Woods M, Buzon M, **Li C**, Pereyra F, Crosby SD, Rychert J, Church G, Martinez-Picado J, Rosenberg E, Telenti A, Yu X, Lichterfeld M. Transcriptional profiling of CD4 T cells identifies distinct subgroups of HIV-1 elite controllers. *Journal of Virology*, Vol. 85 (6): 3015-3019; March 2011.

Brumme Z\*, **Li C\*** (\***co-first author**), Miura G, Sela J, Rosato P, Brumme C, Markle T, Martin R, Block B, Trocha A, Kadie C, Allen T, Pereyra F, Heckerman D, Walker B and Brockman M. Reduced Replication Capacity of NL4-3 Recombinant Viruses Encoding Reverse Transcriptase–Integrase Sequences From HIV-1 Elite Controllers. *Journal of Acquired Immune Deficiency Syndromes*. Vol. 56(2):100-108; February, 2011.